A role for UV-B -induced DNA damage in photomorphogenic responses in etiolated *Arabidopsis* seedlings

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Abstract

Ultraviolet (UV) radiation is a constituent of sunlight that influences plant morphology and growth. It induces photomorphogenic responses but also causes damage to DNA. Plant responses to DNA damage caused by UV-B light are often categorized as general mechanisms that get activated by other environmental stresses. Photodimers are formed through the direct absorption of UV-B light by DNA and are removed, in part, by nucleotide excision repair (NER). UV-B irradiation resulted in the accumulation of the two most common photodimers, cyclobutane pyrimidine dimers (CPDs) and pyrimidine-(6,4)-pyrimidinone dimers (6,4PPs), in etiolated wild type (wt) *Arabidopsis* seedlings. *Arabidopsis* mutants of the endonucleases that function in NER, *xpf-3* and *uvr1-1*, show hypersensitivity to UV-B (280-320 nm) in terms of hypocotyl growth inhibition. I hypothesized that the accumulation of UV-B-induced photodimers was responsible for the hypocotyl growth phenotype of these NER mutants after UV-B irradiation. It was also predicted that the accumulation of photodimers could ultimately trigger signaling pathways that result in cell-cycle arrest through stalled replication sites or double-strand breaks. This was tested using the *suppressor of gamma 1* (*sog1-1*) mutant, which lacks a transcription factor responsible for gene induction and cell-cycle arrest after gamma irradiation, and a Col-0 line containing a CYCB1;1-GUS reporter construct. *CYCB1;1* encodes a cyclin that accumulates in response to cell-cycle arrest at the G2/M transition. The main conclusion from this work is that hypocotyl growth inhibition induced by UV-B light in etiolated *Arabidopsis* seedlings, which is a classic photomorphogenic response, is influenced by signals originating from UV-B light absorption by DNA that lead to cell-cycle arrest. Furthermore, this process is shown to occur independently of *UVR8* and its signaling pathway responsible for *CHS* induction. This work also demonstrates that UV-B-induced DNA damage can be responsible for specific photomorphogenic responses, at least in etiolated *Arabidopsis* seedlings, and does not simply induce general stress responses.

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Preface

Research regarding ultraviolet-B (UV-B) light effects in plants has been a long and complicated story. I began my graduate research work with the goal of characterizing physiological responses in plants to UV-B, specifically to determine how the absorption of UV-B by DNA and subsequent damage products and repair processes influence these responses. A few years later, the identity of a UV-B photoreceptor in plants was revealed to be UVR8, a component that was known to function in UV-B -specific signaling. This paved the way for a wealth of subsequent research dealing with elucidating the actual photoreceptor capacity and mechanism of this molecule as well as defining more thoroughly "THE" UV-B photoreceptor pathway. However, it seemed to me that the idea of how plants could perceive UV-B light and initiate photomorphogenic responses had focused to the UVR8 pathway alone.

Photoreceptors are important molecules in plants that absorb light at specific wavelengths and ultimately influence growth. This modification of growth and development that is dependent on light is called photomorphogenesis. Photoreceptors are typically proteins coupled with a chromophore that absorbs the light and passes an electron to the protein molecule to affect a change. UV-B light is unique, in that it can be absorbed by several different molecules that exist in cells, including DNA, RNA, lipids, proteins, etc. Therefore, the UV-B photoreceptor remained elusive for some time and led to alternative ideas about the perception of UV-B light in plants. The ultimate affect of the absorption of any photon of light involved in photomorphogenesis is a chemical reaction induced by that photon, meaning that if photochemistry happens after the absorption of that photon, photoreception has occurred and downstream processes may be initiated. This is the main argument that underlies the work presented in this thesis.

The research presented here describes a potential UV-B -specific signaling pathway resulting in cell-cycle arrest that is initiated by the direct absorption of a UV-B photon by DNA. This pathway affects a photomorphogenic response that is independent of the identified UV-B photoreceptor, UVR8. A comprehensive review is first provided to highlight the idea that multiple UV-B perception mechanisms exist in plants and to

provide context for the subsequent chapter providing research evidence that the absorption of UV-B light by DNA can affect photomorphogenic growth.

Chapter 1

Multiple UV-B Perception Mechanisms in Plants

Introduction

Plants are dependent on a wide array of environmental signals to modulate growth and morphology and have evolved sophisticated systems for perceiving and responding to such stimuli. Among these is the perception of light signals through photoreceptors that absorb light at specific wavelengths. UV-B light (280-320 nm) is an especially important component of sunlight. It has the highest energy of the solar spectrum that reaches the earth's surface, making it a unique light stimulus because, while it causes damage to biomolecules such as DNA (Britt, 2004; Taylor, 2006), it also induces classic photomorphogenic responses like hypocotyl growth inhibition (Ballaré *et al*., 1991; Kim *et al*., 1998; Shinkle *et al*., 2004; Gardner *et al.,* 2009), cotyledon expansion (Boccalandro *et al.,* 2001), and leaf development (Brown and Jenkins, 2008; Wargent *et al*., 2009) among others (reviewed in Frohnmeyer and Staiger, 2003; Ulm, 2006). Tremendous progress has been made in defining UV-B -specific signaling pathways in plants as well as possible perception mechanisms, and this progress has been extensively reviewed (Brosché and Strid, 2003; Ulm, 2006; Jenkins, 2009; Heijde and Ulm, 2012; Tilbrook *et al*., 2013).

DNA damage caused by UV-B and responses to UV-B are well known. In plants these responses have, historically, been considered non-photomorphogenic or as general responses in plants because they are also activated by other stimuli (reviewed in Brosché and Strid, 2003; Frohnmeyer and Staiger, 2003). Recently, identification of the UVR8 protein as a UV-B photoreceptor in plants (Rizzini *et al*., 2011) has narrowed the view of how plants perceive UV-B light with focus being drawn mainly to this photoreceptor system. However, several methods of UV-B light detection have been defined, at least in human cell lines (Kulms and Schwarz, 2002). While plants and humans are obviously very different, they both contain the same basic cellular components that can absorb UV-

B light. The existence of several UV-B -specific signaling pathways that are independent of the UVR8 photoreceptor suggests that other perception mechanisms exist in plants as well. They are recognized in the literature to some extent, but often ill-defined by the categorical restrictions used to separate the responses. The distinction between "photomorphogenic" and "damage" responses may be helpful for distinguishing the varied effects of UV-B irradiation in plants, but are perhaps not entirely accurate. Photomorphogenesis is development mediated by light (Briggs and Olney, 2001). If signals originating from DNA after absorption of UV-B light ultimately converge to regulate processes, such as gene expression or the cell cycle, then development or growth is affected and photomorphogenesis has occurred. This review focuses on the initial perception of UV-B light in plants that induces downstream processes that ultimately affect growth. In particular, UV-B -induced DNA damage and DNA damage responses will be discussed within the context of being a possible pathway for regulating early photomorphogenesis in plants in response to UV-B light (Fig. 1).

Historical UV-B Research in Plants

Although the impacts of solar UV on plant growth have interested scientists for over a century (reviewed in Caldwell, 1971), research focus on increased UV-B fluxes and their effect on plants was prompted by concerns over decreasing stratospheric ozone, initially discovered in the 1980's (Farman *et al*., 1985). This was a concern because stratospheric ozone is the main barrier to the earth's surface of solar UV radiation. It is most efficient at absorbing higher energy wavelengths (< 290 nm), by essentially excluding UV-C and a small portion of UV-B. UV-A and the remaining UV-B spectrum are transmitted through the ozone layer; however, the UV-B wavelengths (290-320 nm) are greatly reduced (Ulm, 2006). Therefore, as stratospheric ozone levels decrease, the result is not only higher fluxes of those wavelengths that already pass through, but the transmission of shorter wavelength UV-B as well (Caldwell and Flint, 1994). It was known from human based research and associated model systems that DNA damage from UV-B was a primary source of skin cancer (Setlow, 1974). By extension, this was a

potential issue for plants because DNA damage caused by the absorption of UV-B light could inflict cellular damage and decrease overall plant growth and productivity.

Banning the use of chlorofluorocarbons (CFCs) has helped alleviate the large loss of stratospheric ozone over Antarctica each year (Crutzen and Oppenheimer, 2008), but global levels of stratospheric ozone are in an overall decline (NASA, 1999; Forster *et al*., 2010). Interactions with greenhouse gases and other chemicals also make it difficult to predict changes in stratospheric ozone and what future levels will be (Weatherhead and Andersen, 2006). Therefore, increased UV-B radiation at the earth's surface is still a concern, and understanding how plants perceive UV-B light is additionally important, regardless of possible increased fluxes, because it is an inherent component of sunlight and an environmental stimulus for plants.

UV-B Perception in Plants and Its Effects

The effects of UV-B light in plants are varied. Direct absorption of UV-B light by several cellular components leads to downstream effects or responses either directly through that absorption or through indirect consequences. These early effects can manifest in a variety of morphological responses where decreased plant height and biomass accumulation are commonly observed (Jansen *et al*., 1998; Kakani *et al*., 2003; Ballaré *et al.,* 2011). The inhibition of hypocotyl elongation is a classic photomorphogenic response (Beggs *et al*., 1980) and used to gauge sensitivity to UV-B light (Kim *et al*., 1998; Shinkle *et al*. 2004; 2005; Gardner *et al*., 2009). UV-B light induces the expansion of cotyledons and can cause curling in the cotyledon (Boccalandro *et al*., 2001). It also alters leaf expansion and growth (Hopkins *et al*., 2002; Wargent *et al*., 2009).

Several genes that encode enzymes in the phenylpropanoid pathway are strongly induced after UV-B irradiation, and the accumulation of flavonoids and anthocyanins helps plants shield UV-B before reaching other cellular components (Robberecht and Caldwell, 1978; Li *et al*., 1993; Stapleton and Walbot, 1994; Mazza *et al*., 2000). For example, *uvr8* mutants exhibited lower photosynthetic efficiency due to increased photoinhibition from UV-B irradiation, presumably because they lack flavonoids to

screen UV-B light and protect the photosynthetic apparatus. The same *uvr8* plants were severely dwarfed and necrotic compared to wt (Davey *et al*., 2012). Therefore, increased levels of UV-B may have a significant impact on plant growth, especially if they lack sufficient screening compounds. Plant productivity was an initial concern because UV-B irradiation mainly showed effects such as photosynthetic damage, reactive oxygen species (ROS) production, and both direct and indirect DNA damage (reviewed in Jansen *et al.,* 1998).

The majority of UV-B irradiation effects in plants observed under laboratory conditions are unlikely to occur in nature (*e.g.*, UV-C irradiation, artificially high UV-B fluences beyond projected increases, etc.), sparking debate as to what effects are relevant to plants under natural environmental conditions. For example, photosynthetic rates in plants grown under natural conditions have not been shown to be significantly different under changes in UV-B radiation, and therefore do not explain the observed plant growth decreases (Ballaré *et al.,* 2011). Studies using pea suggested that reductions in leaf area and biomass after UV-B exposure were the result of a decrease in cell divisions and smaller cell area (González *et al*., 1998; Nogués *et al*., 1998), providing evidence that growth inhibition can occur through alterations in cell cycle regulation.

Early hypotheses regarding the perception of UV-B light in plants recognized the possibility of multiple pathways that were likely linked to certain wavelengths due to the dependency of biological responses to particular ranges of UV. When action spectra were normalized to the most effective wavelengths, DNA was the main potential chromophore for a majority of the responses (Caldwell, 1971), and more recent work has provided evidence that DNA could be a sensor for photomorphogenic UV-B responses at shorter wavelengths (Shinkle *et al*., 2004; Shinkle *et al*., 2005). However, shorter wavelengths of UV-B (~280-300 nm) are often regarded as "damaging" because of the higher energy associated with them (Ulm *et al*., 2006), so the idea that DNA acting as a specific sensor for UV-B light is not widely accepted. This is because formation of ROS, DNA damage, or lipid peroxidation by ROS are generally attributed to short wavelength UV-B, and these effects can ultimately trigger pathways responsive to other environmental stresses

like wounding or pathogen attack (reviewed in Frohnmeyer and Staiger, 2003; Brosché and Strid, 2003).

Specific UV-B effects that lead to photomorphogenic responses, such as hypocotyl growth inhibition, cotyledon expansion, leaf elongation, or flavonoid biosynthesis are typically induced by longer wavelengths (≥ 300 nm). Because of this distinction, most studies involving UV-B photomorphogenesis now routinely filter out wavelengths lower than 300 nm, which may provide a limited view of how plants actually respond to the full, natural UV-B spectrum. In addition to wavelength dependence, certain responses are fluence-dependent (Kim *et al.,* 1998; Boccalandro *et al.,* 2001; Shinkle *et al*., 2004; Kalbina and Strid, 2006; Brown and Jenkins, 2008), where responses to lower fluence rates are photomorphogenic and responses at higher fluences are considered stress-like. Regardless of specific categorizations of UV-B responses in plants, it is clear that plants perceive UV-B light signals via multiple mechanisms either directly or indirectly, and the initial signal is the absorption of UV-B light.

Direct UV-B Light Absorption in Plants

UV-B light is directly absorbed by a number of components in the cell, including proteins and nucleic acids (Britt, 2004). It is important to distinguish that the direct absorption referred to here is the absorption of UV-B photons that causes the excitation of electrons resulting in rearrangements of molecules (Clayton, 1970). This includes conformational changes in proteins that can be reversed, and is distinct from ionizing radiation like UV-C, gamma, or X-rays, which has enough energy to release electrons from molecules, likely resulting in permanent changes.

The direct absorption of UV-B light by DNA is especially critical due to the formation of photodimers (discussed in more detail below) that create distortions in the DNA strand that block transcription and replication. Unrepaired photodimers can lead to mutations that threaten genome integrity as well as overall plant growth (Ries *et al*., 2000). Consequences of damage products produced in RNA or through the direct absorption of UV-B light by cellular proteins is unknown and not an extensively studied area. One particular protein that absorbs UV-B light directly is UV RESISTANCE

LOCUS 8 (UVR8), and it was recently identified as a UV-B photoreceptor in plants (Rizzini *et al*., 2011) that controls the transcriptional induction of genes involved in the production of flavonoids and other genes regulated by the transcription factor ELONGATED HYPOCOTYL 5 (HY5) (Brown *et al*., 2005). Flavonoids produced in the epidermis of leaves in response to UV radiation presumably absorb UV-B light directly to screen the radiation before it damages cellular components in deeper layers (Robberecht and Caldwell, 1978; Li *et al*., 1993; Stapleton and Walbot, 1994). But flavonoid absorption *per se* is not informational, in that the energy from the absorbed UV-B photon is captured within the molecule (Edreva, 2005) and not used to affect downstream processes or growth.

UV radiation can directly activate cell membrane receptors involved in apoptosis in human cells (Kulms and Schwarz, 2002). Most work in plants has shown that mitogen-activated protein kinase (MAPK) signals are initiated by UV-B, suggesting the involvement of receptors outside the nucleus on the cell surface that are directly activated by UV-B light (Stratmann, 2003; Ulm, 2004). This further demonstrates that the perception of UV-B in plants involves several mechanisms directly activated through UV-B light absorption and is not limited to absorption by the photoreceptor UVR8.

Indirect Effects of UV-B Light Absorption in Plants

Perception of UV-B light also occurs via indirect consequences. Disruption of photosynthetic processes is a common indirect affect of UV-B light exposure (Bornman, 1989; Day and Vogelmann, 1995; A.-H.-Mackerness *et al*., 1997). Photosynthetic electron transport is mainly inhibited through degradation of the D1 and D2 proteins of photosystem II (PSII) after UV-B irradiation (Jansen *et al*., 1996; Vass *et al*., 1996). However, photoinhibition can also occur through damage to PSI (Powles, 1984) and has recently been implicated as a potential source of ROS (Takahashi and Murata, 2008). ROS production is a common observation after UV-B irradiation in light-grown plants (Dai *et al*., 1997; A.-H.-Mackerness *et al*., 1998). Their main effects are on membranes through lipid peroxidation, but they can also oxidize proteins, RNA, and DNA, and critical levels of the oxidation products will eventually lead to cell death (Mittler, 2002).

ROS also function as systemic signals for several environmental stimuli, but this signal has not been documented in response to UV-B irradiation directly (Miller *et al*., 2009). However, one previous study showed that an NADPH oxidase expressed in leaves of *Arabidopsis* was required for full induction of *CHALCONE SYNTHASE* (*CHS*) after UV-B irradiation (Kalbina and Strid, 2006). In addition, ROS causes reduced transcription of photosynthetic genes and increased antioxidant enzyme activity (Krizek *et al*., 1993; Rao *et al*. 1996; Surplus *et al*., 1998). Because of these links to gene expression changes, it is likely that a UV-B-induced systemic signaling pathway for ROS exists in plants (A.-H.-Mackerness, 2000).

Chalcone synthase (CHS) catalyzes the first reaction devoted to flavonoid biosynthesis, and its gene expression is strongly up-regulated by UV-B irradiation. Accumulation of flavonoids and anthocyanins is a common response to UV-B light exposure in plants. *UVR8* is required for the synthesis of flavonoids specifically after UV-B irradiation through the transcriptional induction of *CHS* and other biosynthetic genes involved in the phenylpropanoid pathway (Brown *et al*., 2005). A suite of phenylpropanoid compounds accumulates in response to several environmental stresses such as herbivory, pathogen attack, or low temperatures (Dixon and Paiva, 1995). Although there is UV-B -specific flavonoid and anthocyanin production, synthesis of these molecules occurs after visible light exposure as well, as evidenced by *CHS* induction by blue and red light (Frohnmeyer *et al*., 1992; Christie and Jenkins, 1996).

DNA damage, such as photodimers created by the direct absorption of UV-B light or oxidation products due to interactions with ROS as the result of UV-B irradiation, activate repair processes. Photodimers can be directly reversed through photoreactivation with exposure to blue/UV-A light (Sancar, 1994), which is a unique process to this type of DNA damage. There are also general mechanisms like nucleotide excision repair (NER) or homologous recombination that repair all types of DNA damage. An accumulation of any unrepaired damage will eventually trigger DNA damage responses (discussed in more detail below) that ultimately limit growth through disruption of the cell cycle to allow for repair. In some cases, enough damage has been shown to lead to

apoptosis in plant meristems (Fulcher and Sablowski, 2009; Adachi *et al*., 2011), and it can be specific to UV-B (Furukawa *et al*., 2010).

UV-B Induced DNA Damage and Its Consequences

When DNA absorbs UV-B light directly, energy from the photons cause covalent linkages between adjacent pyrimidine bases creating two main photoproducts, cyclobutane pyrimidine dimers (CPDs) and pyrimidine-6,4-pyrimidinone dimers (6,4PPs). Further exposure to UV irradiation causes photoisomeration of 6,4PPs into the Dewar photoproduct (Mitchell 1988; Takeuchi *et al*. 1998). In humans, DNA is the main UV-B light chromophore, and DNA damage is the source of several downstream effects such as sunburn and skin cancer (Kulms and Schwarz, 2002). In addition, CPDs are thought to be the basis of melanin production (Parrish *et al*., 1982). Plants do not develop cancer (Doonan and Sablowski, 2010), but disruption of the cell cycle can occur in response to UV-B -specific DNA damage (Jiang *et al*., 2011; Biever *et al*., 2013). Furthermore, programmed cell death can be activated if DNA damage accumulates (Fulcher and Sablowski, 2009; Furukawa *et al*., 2010). Plants synthesize flavonoids to absorb UV-B light and limit damage to cellular components (Stapleton and Walbot, 1994) and is somewhat analogous to melanin production in humans. However, photodimers themselves have not been shown to induce flavonoid or anthocyanin production (Hada *et al*., 1996), which is the case for melanin (Parrish *et al*., 1982).

CPDs are by far the most abundant dimers and are produced ~10x more efficiently than 6,4PPs (Taylor, 2006). UV-C light can reverse CPDs, but they do not absorb UV-B light, making them fairly stable in natural light conditions (Taylor, 2006). On the other hand, 6,4PPs absorb maximally at 325 nm and are much less stable in sunlight (Taylor, 2006). The 6,4PP is quite efficiently converted to the Dewar photoisomer with UV light at 325 nm, and both photodimers are rapidly removed by photoreactivation or NER (Mitchell, 1988; Takeuchi *et al*., 1998). Other studies have shown that CPD repair may occur preferentially in the light, whereas 6,4PP repair is more rapid in the dark (Britt, 1993).

The consequences of photodimers, specifically, include the induction of repair mechanisms, stalled replication or transcription sites if not repaired, and downstream DNA damage signaling pathways, all of which are initially set into action from the direct absorption of UV-B light by DNA. There have been no reports of strong transcriptional responses due to UV-B induced photodimer formation like those that occur after gamma irradiation from double-strand breaks and stalled replication sites (Culligan *et al*., 2006; Yoshiyama *et al*., 2009). Since these are ultimate consequences of UV-B irradiation as well, it seems possible that transcriptional responses induced by gamma irradiation would also be induced by UV-B irradiation, a more biologically relevant environmental signal. UV-B irradiation and induced photodimers, however, have been linked to altered expression of cell cycle regulatory genes (Jiang *et al*., 2011).

Repair of Photodimers

Plants are well equipped to cope with DNA damage and have evolved efficient repair mechanisms because they cannot simply move to avoid harmful radiation from the sun. They have two main repair mechanisms for photodimers: photoreactivation and nucleotide excision repair (NER). Photoreactivation occurs only for UV-B photodimers and not other types of damage. CPD or 6,4PP specific enzymes called photolyases reverse photodimer formation and restore the original bases using energy from UV-A or blue light (Sancar, 1994). Photoreactivation is largely why plants are so efficient at repairing photodimers because the photolyase enzymes directly bind and reverse the photodimer through the use of energy from a UV-A/blue light photon. Therefore, this method is the more favorable for photodimer repair because an error that may result in a mutation is less likely to occur. Plants contain two different photolyases that specifically bind either CPDs or 6,4PPs but not both. An enzyme specific for Dewar photoproducts has not been identified. Expression of the CPD photolyase (PHR1) is induced by white light or UV-B, but the 6,4PP photolyase (UVR3) is constitutively expressed (Chen *et al*., 1994; Waterworth *et al*., 2002). The CPD photolyase appears to be regulated by HY5, under control of the UVR8 photoreceptor signaling pathway (Brown *et al*., 2005; Brown and Jenkins, 2008). Recent work has shown that both photolyase genes are under transcriptional control by HY5/HYH and induced upon light exposure (Castells *et al*.,

2010), but the requirement for UVR8 was not tested. There is little repair of CPD photodimers in the dark (Britt *et al*., 1993), and light-dependent repair seems to be the dominant pathway for their removal (Chen *et al*., 1994). In contrast, 6,4PPs are more efficiently removed in the dark via NER, rather than through photoreactivation (Britt *et al*., 1993). However, this may not be the case for all plant species (Hada *et al*., 1996).

Nucleotide excision repair (NER) is a more universal DNA repair mechanism that repairs other DNA damage products in addition to UV-B photodimers. It functions without the need for light energy, and several enzymes are involved (Table 1), resulting in the excision of a small strand of bases flanking, and including, the photodimer. The remaining gap is filled by the normal replication components. This method of repair is considered to be more "error-prone" because it must refill a gap of about 30 nucleotides and disrupts more of the original DNA strand. It can occur throughout the genome as global genomic repair (GGR) or as a more directed process coupled with transcription (TCR; Britt 2002). Most of the information regarding the mechanism of NER has been worked out in other systems such as *E. coli* or yeast (Sancar and Smith, 1989; Wang *et al*., 1993; You *et al*., 2003), but the majority of the genes that encode the enzymes involved are widely conserved among species, including plants. Knowledge regarding the biochemistry of the NER pathway in plants remains limited and is assumed to function similarly to what has been described for other systems (Sugasawa *et al*., 2001; Volker *et al*., 2001). However, there has been limited work using *Arabidopsis* cell extracts to study DNA repair (Li *et al*., 2002).

Both mechanisms contribute to plant tolerance of UV-B light. *Arabidopsis* mutants of the photolyases and NER enzymes are hypersensitive when irradiated with UV-B or UV-C by displaying necrosis and decreased growth (Britt *et al.,* 1993; Harlow *et al*., 1994; Jiang *et al.,* 1997; Landry *et al.,* 1997; Liu *et al*., 2000; Liu *et al.,* 2001). Mutations in the 5'- and 3'-endonucleases involved in NER, in particular, seem to have the most dramatic effect on *Arabidopsis* growth under UV-B (Britt *et al.,* 1993; Harlow *et al.,* 1994; Gardner *et al.,* 2009; Biever *et al*., 2013). However, NER components usually have roles in other types of damage repair because they ultimately recognize single-stranded DNA at stalled replication or transcription sites or other components involved in

those processes (Kunz *et al*., 2005). This means that mutations of NER components may lead to general growth consequences, so when plants are exposed to UV-B light, it is not surprising that those mutants are especially sensitive.

Homologous recombination (HR) seems to, in part, be responsible for the removal of CPDs (Ries *et al*., 2000a; Ries *et al*., 2000b), but not 6,4PPs. UV-stimulated homologous recombination (HR) activity was shown to be proportional to the amount of CPDs formed and dependent on photosynthetic active radiation but independent of the CPD photolyase (Ries *et al*., 2000b). CPDs are formed at a much higher frequency than 6,4PPs, and this may be the reason they are the main photodimer targeted for HR (Ries *et al*., 2000b). However, a lack of data linking HR events to 6,4PPs cannot exclude HR as a possible repair mechanism for this photodimer as well. HR is likely a more secondary process for removal of photodimers. A study using a mutant lacking the CENTRIN2 protein, which stabilizes the photodimer recognition complex involved in NER, showed increased HR (Molinier *et al*., 2004), indicating that HR is more prominent only when other repair processes are inhibited.

DNA Damage Response Signaling Pathways

The detection of DNA damage is an important process for resistance and tolerance to environmental factors causing damage, such as UV-B light (Culligan *et al.,* 2004). An elaborate network of proteins is employed to recognize the damage and initiate a signaling cascade that inhibits progression of the cell cycle to limit the proliferation of potential mutations. This network is a conserved response among several organisms (Melo and Toczyski, 2002) and activated through the recognition of double-strand breaks or replication forks (single-stranded DNA) by the protein kinases ATAXIA-TELANGIECTASIA MUTATED (ATM) and ATM AND RAD3-RELATED (ATR), respectively (Garcia *et al.,* 2003; Culligan *et al.,* 2004). The accumulation of unrepaired UV-B-photodimers results in stalled replication sites and, to a lesser extent, double strand breaks (Molinier *et al*., 2004) and, therefore, can activate DNA damage responses. SUPPRESSOR OF GAMMA 1 (SOG1) is a plant-specific transcription factor in this pathway and was postulated to be analogous to p53 in mammalian systems (Yoshiyama

et al. 2009). SOG1 is necessary for downstream signaling from ATM and ATR and is required for transcriptional responses after gamma irradiation (Preuss and Britt, 2003; Yoshiyama *et al*., 2009). It may also function independently of ATM and ATR pathways in UV-B -specific DNA damage signaling (Biever *et al*., 2013).

Most of the DNA damage response pathway(s) in plants has been determined by studies using gamma irradiation to inflict damage, and the ultimate effect of DNA damage signaling is growth arrest through alteration of the cell cycle. UV-B was shown to induce the same signaling pathways shared by that of gamma irradiation that lead to programmed cell death in the root apical meristem (Furukawa *et al*. 2010). These pathways were also activated in the shoot primordia (Fulcher and Sablowski 2009), but UV-B-induced DNA damage, specifically, was not studied. The existence of these pathways does, however, show that UV-B-induced DNA damage could affect plant growth in this way. Instead of cell-cycle arrest, endoreduplication cycles were initiated in response to double-strand breaks in *Arabidopsis* cells and were regulated through ATM and ATR signaling dependent on SOG1 (Adachi *et al*., 2011). UV light did not induce endoreduplication, but this study was specific to root and sepal cells (Adachi *et al*., 2011). However, endoreduplication may be important in other plant tissues in response to UV-B. It was shown to be an alternative pathway to cell-cycle arrest in leaves and was dependent on UVR8 (Wargent *et al*., 2009). Also, the *uvi4* mutant isolated in *Arabidopsis* underwent additional endoreduplication rounds in the hypocotyl and was less sensitive to UV-B irradiation than the wt (Hase *et al*., 2006), indicating that endoreduplication is a possible consequence of UV-B -induced DNA damage.

Perception of UV-B by UVR8, A UV-B Photoreceptor

The UV-B specific signaling pathway regulated by UV RESISTANCE LOCUS 8 (UVR8) is probably the most characterized mechanism regarding photomorphogenic responses to UV-B in plants. The *uvr8-1* mutant was originally isolated as being more sensitive to UV-B than the wild type when grown in the light (Kliebenstein *et al*., 2002). *uvr8* mutants are deficient in UV-B specific *CHS* induction and also show increased levels of PR1 and PR5 (Kliebenstein *et al*., 2002; Brown & Jenkins, 2008), proteins

involved in stress responses such as defense against pathogens. In addition, UVR8 regulates expression of the transcription factors ELONGATED HYPOCOTYL5 (HY5) and its homolog HYH (Brown *et al*., 2005; Brown and Jenkins, 2008) by directly interacting with CONSTITUTIVELY PHOTOMORPHOGENIC1 (COP1) during UV-B exposure. This interaction inhibits a repressor of UVR8 that is associated with *HY5/HYH* chromatin and allows activation of these transcription factors and subsequent genes under their control (Favory *et al.,* 2009).

Accumulation of UVR8 in the nucleus occurs shortly after UV-B irradiation (Kaiserli and Jenkins, 2007). The mechanism for UVR8 translocation into the nucleus has yet to be determined, but UVR8 also, constitutively and independently of UV-B light, binds to chromatin (Cloix and Jenkins, 2008). UVR8 is mainly located in the cytoplasm, but there is at least a small pool of UVR8 that already exists in the nucleus (Kaiserli and Jenkins, 2007). However, expression of genes regulated by UVR8 requires UV-B exposure (O'Hara and Jenkins, 2012). *UVR8* itself is not induced by UV-B and protein levels remain fairly constant in dark grown compared to light grown plants (Kaiserli and Jenkins, 2007; Rizzini *et al.,* 2011; O'Hara and Jenkins, 2012).

Mechanism for UV-B Perception by UVR8

UVR8 was recently demonstrated to act as a UV-B photoreceptor *in vitro* (Rizzini *et al*., 2011). Early characterization of *UVR8* showed it was homologous to the human gene *REGULATOR OF CHROMATIN CONDENSATION* (*RCC1*), which is a guanine nucleotide exchange factor for the G-protein Ran (Kliebenstein *et al*., 2002), but this activity has not been observed in plants. It interacts with itself to form a dimer whereupon UV-B irradiation *in vitro*, it monomerizes (Rizzini *et al*., 2011). Biochemical analyses demonstrated that tryptophans were required for dimer formation and also formed the chromophore for UV-B absorption (Christie *et al*., 2012; Wu *et al*., 2012). Specifically, a "tyrptophan pyramid" forms between UVR8 monomers and is surrounded by charged and other aromatic residues that create salt bridges at the dimer interface. Monomerization occurs when the cross-dimer salt bridges are disrupted through UV-B light absorption by the tryptophan pyramid (Christie *et al*., 2012). The monomer is the

active form and binds to COP1 to regulate downstream gene expression (Figure 1) (Favory *et al*., 2009; Rizzini *et al*., 2011).

The unique cluster of tryptophans at the center of the protein was originally hypothesized to be required for dimerization and interaction with COP1 because two of the tryptophans that were mutated to alanine lost the ability to form dimers but retained their interaction with COP1 (Rizzini *et al*., 2011). One particular mutation UVR8^{W285A} constitutively interacted with COP1, but did not form dimers. UVR8^{W285F} did form dimers but was unresponsive to UV-B and showed no interaction with COP1 (Rizzini *et al*., 2011). It would seem that the UVR8^{W285A} would show constitutive responses to UV-B that are regulated by UVR8 such as expression of *HY5* or *CHS*, but *in vivo* experiments interestingly showed that these mutants were phenotypically similar to *uvr8* mutants by lacking *HY5* and *CHS* expression and hypocotyl growth inhibition after UV-B irradiation (O'Hara and Jenkins, 2012). Biochemical analysis demonstrated that the UVR8^{W285A} mutant was structurally very similar to the wt UVR8 dimer (Christie *et al*., 2012), which would explain the lack of downstream responses initiated by UVR8 after UV-B exposure previously reported (O'Hara and Jenkins, 2012). Responses, such as constitutive expression of *HY5* and *CHS* would be expected to some degree, as well, based on the results mentioned above regarding UVR8 binding to chromatin independent of UV-B. Whether it was the dimer or monomer that was constitutively bound to chromatin, however, was not specified (Cloix and Jenkins, 2008).

UVR8-independent responses specific to UV-B

There are documented UV-B-specific responses that occur independently of UVR8, demonstrating that UV-B perception in plants must occur via multiple mechanisms. Brown and Jenkins (2008) described a high-fluence rate response in *Arabidopsis* leaves that induced gene expression specifically in response to UV-B irradiation, but did not require *UVR8.* The three genes identified in this category were *WRKY30* (At5g24110), *UDPgtfp* (At1g05680) and *FAD oxred* (At1g26380). Both *UDPgtfp* and *FAD oxred* are known to be up-regulated by H_2O_2 (Inzé *et al*., 2011). Not much is known about *WRKY30* specifically, but *WRKY* transcription factors, in general,

regulate a wide range of plant processes, and they function most notably in plant immunity, defense, and leaf senescence (Pandey and Somssich, 2009; Besseau *et al*., 2012). Because of the implicated functions of these genes and the fact that their expression was observed after irradiation with the highest UV-B fluences tested, it was concluded that this response likely overlaps with oxidative stress or wound signaling pathways (Brown and Jenkins, 2008). The overlap of UV-B -specific signaling with such pathways has been the subject of many studies (reviewed in Brosché and Strid, 2003; Frohnmeyer and Staiger, 2003). However, the idea of UV-B as a stressor has recently been the subject of speculation and perhaps an artifact of irradiation conditions that are unlikely to occur outside the laboratory (Hideg *et al*., 2013).

Signal transduction from several different stress responses converge by activating mitogen-activated protein kinase (MAPK) networks (Holley *et al*., 2003). The signaling network involving MAP kinase phosphatase 1 (MKP1), in particular, is activated by UV-B irradiation and independently of UVR8 (Holley *et al*., 2003; Kalbina and Strid, 2006; González Besteiro *et al*., 2011). The *mkp1* mutant was originally identified by its hypersensitivity in terms of root growth to genotoxic stress caused by UV-C irradiation (Ulm *et al*., 2001). Whether MAPK pathways are activated by UV-induced DNA damage directly, by ROS, or other signals is unknown. However, as discussed above, signaling induced by DNA damage has been shown to occur independently of *UVR8*.

Regulation of UV-B Light Perception and Responses

Plant responses to signals from the environment are ultimately regulated by downstream components that control gene expression or other aspects of growth. The E3 ubiquitin ligase, COP1, is a main regulator of photomorphogenesis, specifically (Deng *et* al. 1991), along with DE-ETIOLATED 1 (DET1; Chory *et al*., 1989) that targets other proteins for degradation. COP1/DET1 are negative regulators of light-mediated development because both mutants display light-grown phenotypes when grown in the dark. COP1's regulation of UV-B photomorphogenesis is different from other types of light as it typically degrades the transcription factor HY5 in the dark, and upon light exposure, is inhibited allowing HY5 to induce transcription of genes under its control

(Oravecz *et al*., 2006; Favory *et al*., 2009). The photoreceptor UVR8 interacts directly with COP1 to promote UV-B photomorphogenesis in plants through transcriptional induction of *HY5*, and therefore, the induction of genes that requires HY5 (Favory *et al.* 2009).

Negative regulation of the UVR8-mediated UV-B signaling has been identified and suggested to be controlled by *RUP1* and *RUP2* (Gruber *et al*., 2010). These REPRESSOR OF UV-B PHOTOMORPHOGENESIS (RUP) proteins are highly homologous to one another and contain WD40-repeats similar to COP1. Each one is transcriptionally induced by UV-B light and dependent on UVR8-COP1 interaction and HY5. However, other types of light induce *RUP1* and *RUP2*, so they may have a more general role in light responses (Gruber *et al*., 2010). Induction of *CHS* after UV-B irradiation is much higher in the *rup2* mutant and is basically abolished in overexpression lines (Gruber *et al*., 2010). *rup1rup2* hypocotyl growth inhibition after UV-B light exposure is much more severe than wild type, but they seem to be more readily acclimated to UV-B (Gruber *et al*., 2010). RUP1/RUP2 regulation of the UVR8-signaling pathway does not appear to be dependent on UV-B irradiation. It was recently suggested that RUP1 and RUP2 physically facilitate UVR8 redimerization after UV-B-induced monomerization that "turns off" UVR8-controlled photomorphogenesis (Heijde and Ulm, 2013).

DNA repair proteins are also under regulatory control by DET1 and COP1. Both DET1 and COP1 regulate the expression of the photolyase genes *PHR1* and *UVR3* by degrading HY5/HYH in the dark. *det1* mutants were more tolerant to UV-C irradiation due to a combined effect of increased expression of the photolyase genes and genes involved in the phenylpropanoid pathway (Castells *et al*., 2010). DET1 is also required for proper nucleotide excision repair function through associations with the photodimer recognition factors DDB2 and CSA that detect conformational changes in the DNA strand or stalled RNA polymerases, respectively (Castells *et al.*, 2011). Both proteins interact with CUL4-DDB1 complexes, which associate with DET1 during normal *Arabidopsis* development and are necessary for UV tolerance (Al Khateeb and Schroeder, 2007; 2009; Biedermann and Hellmann, 2010). The CUL4-DDB1-mediated degradation

of DDB2 was shown to require ATR, indicating regulation is also important for checkpoint responses (Molinier *et al*., 2008). The results of these studies are important because they provide evidence that DNA repair processes and DNA damage signaling are necessary for proper plant development and are under control of DET1 and COP1, major components that regulate photomorphogenesis.

Conclusion

Plant responses to UV-B light are highly varied; therefore, the existence of multiple perception pathways seems logical. While this idea is accepted to some degree, the categorization of plant UV-B responses limits room for interpretation regarding "damage-like" or "photomorphogenic" effects. It seems naive to assume that plants would contain a single photoreceptor system for UV-B light, when plants have redundant or homologous photoreceptors for other light qualities. The objective of this review was to highlight the fact that the absorption of UV-B occurs through a variety of ways and induces responses specific to that absorption. What may be considered traditional photomorphogenic responses can be induced through other perception mechanisms apart from the defined UVR8 signaling pathway.

Table 1. *Arabidopsis* genes involved in nucleotide excision repair (NER) and photoreactivation. The major components involved in damage recognition and early steps of NER, a non-comprehensive list.

Gene Name and Designation	Description/Function	Source
UVH3/UVR1 (At3g28030)	XPG/RAD2 homolog; 3' DNA-specific endonuclease involved in NER	Liu *et al*., 2001
UVH1/XPF (At5g41150)	XPF/RAD1 homolog; 5' DNA-specific endonuclease involved in NER, functions with ERCC1/RAD10	Liu *et al*., 2000
UVR7/ERCC1 (At3g15620)	ERCC1/RAD10 homolog; 5' DNA-specific endonuclease involved in NER, functions with XPF/RAD1	Hefner *et al.,* 2003
UVH6 (At1g03190)	XPD/RAD3 homolog; DNA helicase involved in NER	Liu *et al*., 2003
UVR2/PHR1 (At1g12370)	PHR1, CPD photolyase	Ahmad *et al.,* 1997
UVR3 (At3g15620)	6,4PP photolyase	Nakajima *et al*., 1998
CENTRIN2 (At4g37010; At3g50360)	Modulates NER and homologous recombination (HR) pathways; interacts directly with RAD4	Molinier *et al*., 2004
XPC (At5g16630)	RAD4 homolog; interacts with CEN2 and RAD23 in DNA damage recognition	Liang *et al*., 2006
RAD23 (At1g79650; At1g16190; At3g02540; At5g38740; At5g16090)	HR23A,B homolog; stabilizes DNA damage recognition complex (XPC) in NER	Farmer *et al*., 2010
RPA (At4g19130; At5g45400; At2g06510; At5g61000; At5g08020; At2g24490; At3g02920)	Replication protein A; binds and stabilizes single-stranded DNA	Kunz *et al*., 2005

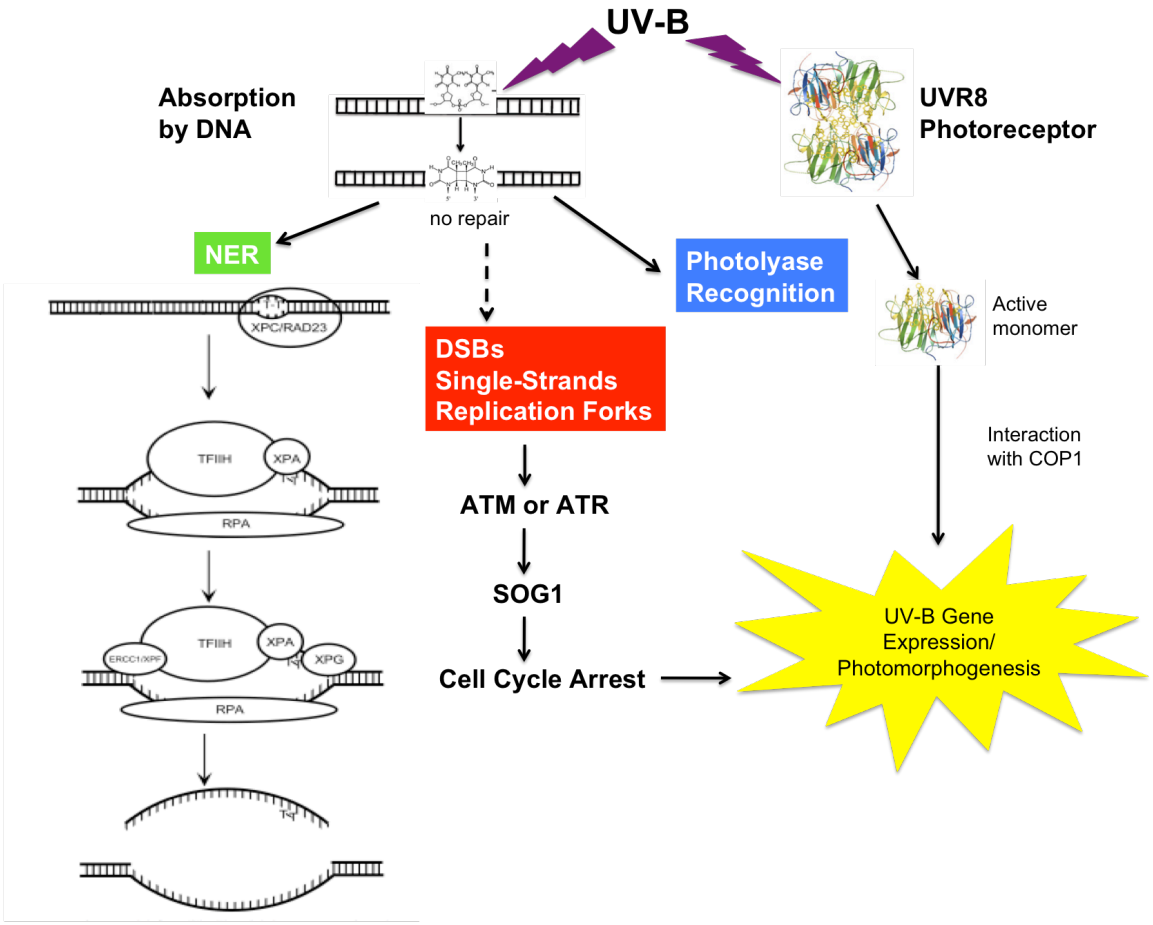

Figure 1. A summary of proposed UV-B perception pathways in etiolated *Arabidopsis* seedlings. UV-B is directly absorbed by the UVR8 photoreceptor. It monomerizes and interacts with COP1 to induce expression of genes under the control of HY5/HYH. Concurrently, DNA directly absorbs UV-B light to form photodimers. Repair processes like NER and photoreactivation can efficiently repair photoproducts to a degree. Cell-cycle arrest is induced by unrepaired photodimers that are either recognized directly, through double-strand breaks (DSBs) or stalled replication sites by the ATM/ATR-SOG1 signaling pathways. Both mechanisms ultimately affect photomorphogensis. (Image of NER pathway appears in Britt, 2004; structures of UVR8 dimer and monomer appear in Heijde and Ulm, 2012)

Chapter 2

UV-B inhibition of hypocotyl growth in etiolated *Arabidopsis thaliana* seedlings is a consequence of cell-cycle arrest initiated by photodimer accumulation[1]

Summary

Arabidopsis mutants of the endonucleases that function in nucleotide excision repair, *xpf-3* and *uvr1-1*, showed hypersensitivity to UV-B (280-320 nm) in terms of hypocotyl growth inhibition. SOG1 is a transcription factor that functions in the DNA damage signaling response after gamma irradiation. *xpf* mutants that carry the *sog1-1* mutation showed hypocotyl growth inhibition after UV-B irradiation similar to wild type. A DNA replication inhibitor, hydroxyurea (HU), also inhibited hypocotyl growth in etiolated seedlings, but *xpf-3* was not hypersensitive to HU. UV-B irradiation induced accumulation of the G2/M-specific cell cycle reporter construct *CYCB1;1-GUS* in wild type *Arabidopsis* seedlings that was consistent with expected accumulation of photodimers and coincided with the time course of hypocotyl growth inhibition after UV-B treatment. Etiolated mutants of *UVR8*, a recently described UV-B photoreceptor, irradiated with UV-B showed inhibition of hypocotyl growth that was not different from wild type, but they lacked UV-B -specific expression of chalcone synthase (*CHS*), as expected from previous reports. *CHS* expression after UV-B irradiation was not different in *xpf-3* than in wild type, nor was it altered after HU treatment. These results suggest that hypocotyl growth inhibition by UV-B light in etiolated *Arabidopsis* seedlings, a photomorphogenic response, is determined by signals originating from UV-B absorption by DNA that lead to cell-cycle arrest. This process occurs distinct from *UVR8* and its signaling pathway responsible for *CHS* induction.

[1] This chapter has been accepted to the *Journal of Experimental Botany, special issue on Photobiology*, January 2014.

Introduction

Plants have evolved sophisticated systems for perceiving and responding to a wide array of environmental stimuli. Among these is the perception of light signals through photoreceptors that absorb light at specific wavelengths. Ultraviolet (UV) radiation is a particularly important part of sunlight that dictates plant morphology and growth. UV-B light (280-320 nm), specifically, is a unique light stimulus in that it induces photomorphogenic responses in plants and also causes damage to biomolecules such as DNA. Many years ago, action spectra of several plant responses to UV irradiation implicated DNA as the main chromophore based on relative photon effectiveness weighted to 280 nm (Caldwell, 1971). However, plant responses to UV-B -induced DNA damage are often considered a general reaction to stress rather than a specific consequence of UV-B light perception (Brosché and Strid, 2003; Frohnmeyer and Staiger, 2003).

When DNA absorbs UV-B light, energy from the photon causes covalent linkages to form between adjacent pyrimidine bases, creating photodimers (Taylor, 2006), primarily cyclobutane pyrimidine dimers (CPDs) and pyrimidine-6,4-pyrimidinone dimers (6,4PPs). Photodimers create such distortions in the DNA strand that they block transcription and replication (Britt, 2004). This is harmful to overall plant growth and genome integrity if they are not repaired (Ries *et al*., 2000), and UV-B photodimers can activate DNA damage response pathways that result in cell-cycle arrest or programmed cell death, at least in stem cells of the root apical meristem (Curtis and Hays, 2007; Furukawa *et al.,* 2010). Fortunately, plants have fairly robust mechanisms to repair photodimers. CPD- or 6,4PP -specific enzymes called photolyases require UV-A/blue light to reverse photodimer formation and restore the original bases (Sancar, 1994). Nucleotide excision repair (NER) is an additional DNA repair mechanism and functions without the need for light energy. Several enzymes are involved, resulting in the excision of a small strand of bases flanking, and including, the photodimer. The remaining gap is filled by the normal replication components. Both mechanisms contribute to plant tolerance of UV-B light. *Arabidopsis thaliana* (*Arabidopsis*) mutants of the photolyases and NER enzymes are hypersensitive when irradiated with UV-B or UV-C (Britt *et al.,*

1993; Harlow *et al*., 1994; Jiang *et al.,* 1997; Landry *et al.,* 1997; Liu *et al*., 2000; Liu *et al.,* 2001). Mutations in the endonucleases involved in NER, in particular, seem to have the most dramatic effect on *Arabidopsis* growth under UV-B (Britt *et al.,* 1993; Harlow *et al.,* 1994; Gardner *et al.,* 2009).

Plants have a UV-B -specific signaling pathway that requires *UV RESISTANCE LOCUS 8* (*UVR8*), and several excellent recent reviews have been published regarding this pathway (Jenkins, 2009; Heijde and Ulm, 2012; Tilbrook *et al*., 2013). Briefly, dimers of UVR8 function as a UV-B photoreceptor (Rizzini *et al*., 2011), and the elegant crystallographic and spectroscopic studies of Christie *et al.* (2012) and Wu *et al*. (2012) demonstrated that the absorption of UV-B by specific tryptophan residues in UVR8 causes dissociation of the UVR8 dimer *in vitro*. Subsequent studies showed that the UVR8 monomer is necessary for interaction with CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1) and downstream transduction though ELONGATED HYPOCOTYL 5 (HY5) *in planta* (O'Hara and Jenkins, 2012). *uvr8* mutants were originally isolated due to their hypersensitivity to UV-B when grown in the light and lack of *CHS* induction and subsequent accumulation of flavonoids compared to wild type (wt) (Kliebenstein *et al*., 2002). However, *uvr8* mutants have also demonstrated insensitive hypocotyl growth inhibition in seedlings to UV-B light (Favory *et al.,* 2009; O'Hara and Jenkins, 2012).

Previous work using etiolated *Arabidopsis* seedlings showed that a mutant of the 3'-endonuclease involved in NER, *uvr1-1*, was more sensitive in terms of hypocotyl growth inhibition than wt after UV-B irradiation (Gardner *et al*., 2009). The same study reported that a mutant of *UVR8* had similar hypocotyl growth inhibition as wt after UV-B irradiation. Based on that work, we hypothesized that UV-B -induced DNA damage, specifically photodimers, leads to hypocotyl growth inhibition in etiolated *Arabidopsis* seedlings. The following experiments show that photomorphogenic inhibition of hypocotyl growth in response to UV-B irradiation in etiolated *Arabidopsis* seedlings is the consequence of cell-cycle arrest activated by the accumulation of UV-B -induced DNA photodimers.

Materials and methods

Plant material

Seeds of the *Arabidopsis* nucleotide excision repair mutant *uvr1-1* (CS8852) were purchased from the Arabidopsis Biological Resource Center (Columbus, OH, USA). *xpf-3*, *xpf sog1-1*, *sog1-1*, and Col:*CYCB1;1-GUS* (Colón-Carmona *et al.* 1999) seeds were generously supplied by A. Britt (UC-Davis, CA, USA). The *uvr8-2* mutant was a gift from G. Jenkins (University of Glasgow, UK). The *uvr8-6* was a gift from R. Ulm (University of Geneva, CH). Wt accessions L*er* and Col-0 were purchased from Lehle Seeds (Round Rock, TX, USA).

Light sources and measurements

UV light sources utilized are as described in Gardner *et al.* (2009). Briefly, broad-band UV-B light (FS40-T12-UVB-BP fluorescent tubes, UV Lighting Co., Brook Park, OH, USA) was used for initial fluence response analyses. Monochromatic UV-B light was supplied by a 100 W xenon arc lamp through a UV grating monochromator (Newport Corporation, Stratford, CT, USA). This was used for gene expression assays and later fluence-response curves. Fluence rates (μmol m^{-2} s^{-1}) for both light sources were measured using a model UVM-SS UV Meter (Apogee Instruments, Logan, UT, USA). Total fluence values (μmol m^{-2}) were achieved by varying the time of irradiation. Blue light for photoreactivation was provided by a Heliospectra L1 prototype LED light source (Heliospectra AB, Göteborg, Sweden) using only the 400 nm LEDs. Fluence rate at the level of the plants was approximately 2.5 μmol m^{-2} s^{-1}, measured with an Apogee Model SPEC-UV/PAR spectroradiometer.

Seed germination and growth

All experiments were conducted with etiolated Arabidopsis seedlings. Seeds were germinated and maintained in complete darkness on Whatman #1 filter paper in 60 mm x 15 mm plastic Petri dishes with 0.5X strength Murashige and Skoog (1962) medium supplemented with 100 μM GA_4 (Valent Biosciences, North Chicago, IL, USA), herein referred to as MS/GA_4 solution. Treatments, either UV-B or chemical, were always

applied shortly after germination when seedlings were about 1-2 mm long, approximately two to three days after planting.

Inhibition of hypocotyl elongation by UV-B

Fluence-response curves for the inhibition of hypocotyl elongation by UV-B were conducted as described in Gardner *et al.* (2009) with minor adjustments. Seeds were germinated as described above with 330 µL MS/GA_4 solution. Two- to three-day-old etiolated seedlings were irradiated with either broad-band (FS40-T12-UVB-BP fluorescent tubes, 10.2 µmol $m^{-2}s^{-1}$) or monochromatic UV-B (290 nm, 3.2 µmol $m^{-2}s^{-1}$); the desired fluence was achieved by varying the duration of the radiation. The seedlings were returned to darkness for two additional days and then transferred to a glass plate and digitally photographed. Hypocotyl lengths were measured using ImageJ software (http://rsb.info.nih.gov/ij/).

Photodimer detection

Two- to three-day-old etiolated seedlings were irradiated with 10^4 µmol m^{-2} monochromatic UV-B at 290 nm. Seedlings (~100-200) were frozen in liquid nitrogen immediately after irradiation and stored at -80 °C. DNA was extracted with a Qiagen DNeasy Plant Mini Extraction kit. All DNA samples were diluted to 0.2 ng/µL with phosphate buffered saline (pH 7.2). An enzyme-linked immunosorbent assay (ELISA) was performed in a 96-well microtiter plate using monoclonal antibodies specific for either CPDs (TDM-2) or 6,4PPs (64M-2) (MBL International Corporation, Woburn, MA) on 10 ng of DNA following the manufacturer's protocol with additional modifications from Mori *et al*. (1991). CPD and 6,4PP content was determined by measuring absorbance at 492 nm of six replicates from each DNA sample using a SpectraMax 190 microplate reader (Molecular Devices, LLC, Sunnydale, CA, USA).

Hydroxyurea treatment

Dose-response curves for the inhibition of hypocotyl elongation by the radiomimetic agent hydroxyurea (HU) were conducted similarly as UV-B fluence-

response curves; however, 300 µL of the MS/GA_4 solution was used for germination. Two- to three-day-old etiolated seedlings were treated with HU over a range of concentrations diluted with 0.5X strength MS (without GA_4) in a total volume of 100 µL. Two days after treatment, hypocotyls were digitally photographed and measured as described above for determining the inhibition of hypocotyl elongation by UV-B. A dish containing seedlings that were not given any additional treatment and one treated with 100 µL 0.5X strength MS medium were used as controls. The concentration of HU that induced a 50% reduction in hypocotyl elongation was 1 mM (Fig. 2A), and this concentration was used for subsequent experiments. When both UV-B and HU were applied, seedlings were first irradiated with UV-B and then given 1 mM HU immediately afterward.

Gene expression

Two- to three-day-old etiolated seedlings (~100-200 per Petri dish) were either irradiated with monochromatic UV-B at 290 nm, given 1 mM HU, or both, and then maintained in the dark until harvest 2-24 h after irradiation. Samples were immediately frozen in liquid nitrogen and stored at -80°C. Total RNA was extracted using a PureLink RNA Mini Kit (Invitrogen) following on-column DNase digestion instructions. Extracts were quantified with a Qubit Fluorometer (Invitrogen) and a Quant-iT BR RNA Assay Kit (Invitrogen). cDNA was synthesized in duplicate from 5 ng of total RNA extracts for each reaction using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories). Duplicate reactions were pooled after synthesis and stored at -20ºC. Real-time reactions were set up in triplicate according to Bio-Rad iQ SYBR Green Supermix instructions and run on the CFX96 Real-Time System (Bio-Rad Laboratories). Gene expression values were automatically calculated by the accompanying CFX Manager 2.0 software using a Livak $2^{-\Delta\Delta CT}$ method and *ACTIN2* (At3g18780) as the reference gene. Primer sequences used were: *ACTIN2* (At3g18780) *ACTIN* Fwd 5'-GTT GGG ATG AAC CAG AAG GA-3', *ACTIN* Rev 5'-GCT CTT CAG GAG CAA TAC GAA G-3'; *CHS* (At5g13930) *CHS* Fwd 5'-CCT GAC ACA TCT GTC GGA GA-3', *CHS* Rev 5'-GGT GAG ACC AAC TTC CCT CA-3'; *UDPgtfp* (At1g05680) *UDP* Fwd 5'-CTG GAG TCC TCA GCT TGA

CGT A-3', *UDP* Rev 5'-TCA CCT TCT GCC TTA ACC CTT A-3'; *CYCB1;1* (At4g37490) *CYCB1;1* Fwd 5'-CCT CGC AGC TGT GGA ATA TGT-3', *CYCB1;1* Rev 5'-TCA ACC ACT CCA CCA GGA TCA-3'.

CYCB1;1-GUS staining

Two- to three-day-old etiolated seedlings containing a *CYCB1;1-GUS* reporter construct (Colón-Carmona *et al.,* 1999) were irradiated with broad-band UV-B and harvested immediately (0 h) or at various times up to 48 h after irradiation. Each time point had a corresponding dark or un-irradiated control. During harvest, approximately ten seedlings were placed in 5 mL of staining solution (100 mM disodium phosphate pH 7.0, 1 mM X-GlcA, 5% sodium azide) for each sample and incubated at 37ºC for 2 days. Seedlings were destained with 70% ethanol for 1 day at 65ºC. GUS-expression was visualized using light microscopy. Each experiment was repeated twice and representative seedlings are shown.

Results

Nucleotide excision repair mutants are hypersensitive to UV-B

Previously, Gardner *et al.* (2009) tested hypocotyl growth inhibition by UV-B in DNA repair mutants and found that *uvr1-1*, a mutant deficient in the 3'-endonuclease involved in NER, was an order of magnitude more sensitive than wt (L*er*). Here, hypocotyl growth inhibition by UV-B in *xpf-3,* a mutant deficient in the 5'-endonuclease of NER was also hypersensitive to UV-B compared to its Col-0 wt. The inhibition of hypocotyl growth of both NER mutants was greatly increased, about 70% from dark controls, at the lowest UV-B irradiation treatments (fluences) tested. This is compared to wt that had approximately 10-20% and 50-55% growth inhibition compared to dark controls at $3x10^3$ μmol m^{-2} and 10^4 μmol m^{-2}, respectively (Fig. 1A). Hypocotyl lengths of the etiolated seedlings are similar before irradiation, and *xpf-3* seedlings are visibly much shorter after 10^4 μmol m^{-2} UV-B (Supplementary Fig. S1). The UV-B response of NER mutants suggests that an accumulation of unrepaired DNA damage, specifically photodimers, affects early hypocotyl growth in etiolated *Arabidopsis* seedlings. In Col-0

wt, photodimer content of both cyclobutane pyrimidine dimers (CPD) and pyrimidine-6,4-pryimidinone dimers (6,4PP) increased after UV-B irradiation at 290 nm compared to the dark control (unirradiated) samples (Fig. 1B). This coincided with the approximately 40% reduction in hypocotyl growth after the same irradiation treatment (Fig. 1C). Furthermore, blue light (BL) treatment either before or concurrent with UV-B irradiation reversed the hypersensitivity of *xpf-3* to UV-B irradiation alone (Fig. 1D). This suggests that the increased hypocotyl growth inhibition of *xpf-3* is a photoreactivatable response and a consequence of photodimer accumulation. In addition, wt and *xpf-3* are completely viable after UV-B treatment once transferred to white light and then to soil (Supplementary Fig. S2).

sog1-1, a gamma irradiation insensitive mutant, reverses *xpf-3* hypersensitivity to UV-B

Pruess and Britt (2003) reported that after irradiation with gamma rays, *xpf* mutants showed a strong induction of a subset of genes and had delayed growth due to cell-cycle arrest in response to an accumulation of double-strand breaks and stalled replication sites. In the same report, they isolated *sog1-1* using a screen for mutations that suppressed the gamma irradiation response in *xpf* seedlings (Pruess and Britt, 2003). Because UV-B can cause similar types of DNA damage as gamma irradiation, albeit less efficiently, and plants are not exposed to significant gamma irradiation in nature, it is likely that responses to DNA damage caused by UV-B in *Arabidopsis* lead to delayed growth similar to that induced by gamma radiation. Therefore, inhibition of hypocotyl elongation of *sog1-1* was measured in response to UV-B and was the same as in wt (Fig. 1C). In addition, the double mutant *xpf sog1-1* also exhibited a wt response to UV-B, indicating that *sog1-1* reversed hypocotyl growth inhibition by UV-B in *xpf*, which parallels *sog1-1* reversal of gamma irradiation responses in *xpf*. We have not observed a similar hypocotyl growth reversal in *xpf* that contains *atm* or *atr* mutations, components involved in DNA damage response signaling (Supplementary Fig. S3). In addition, we measured UV-B hypocotyl growth inhibition in other DNA repair mutants, including

rad23, *ddb1a*, *ddb1b*, *uvr7*, *uvr2*, and *rpa* as well as the cell cycle mutant *wee1* and have not observed any differences from wt (data not shown).

The radiomimetic compound hydroxyurea (HU) also induces inhibition of hypocotyl growth in *Arabidopsis* seedlings, but *xpf-3* is not hypersensitive to HU

Since *xpf* mutants have delayed growth after gamma irradiation by arresting the cell cycle, and SOG1 was required (Pruess and Britt, 2003), it is possible that the hypersensitive hypocotyl growth response to UV-B irradiation in *xpf-3* is due to cell-cycle arrest. To determine whether cell-cycle arrest affects hypocotyl elongation as UV-B did, HU was applied to etiolated seedlings. HU inhibits DNA replication, resulting in a cell-cycle block at the G1/S transition (Planchais *et al.,* 2000), and has been used to mimic replication blocks that may result from UV-B or gamma induced DNA damage (Culligan *et al*., 2004; Adachi *et al.,* 2011). In etiolated Col-0 wt seedlings HU inhibited hypocotyl elongation in a dose-dependent manner, with a 50% reduction in hypocotyl growth after 1 mM HU was applied (Fig. 2A). The effect of HU, when given after UV-B irradiation, was not altered after the lower UV-B fluences and was comparable to the hypocotyl growth inhibition after 10^4 μmol m^{-2} UV-B alone (Fig. 2B). However, there was increased inhibition of hypocotyl growth when HU was applied after 10^4 μmol m^{-2} UV-B, compared to that same UV-B fluence alone (Fig. 2B), indicating an additive effect of the UV-B irradiation and HU.

In contrast to its response to UV-B (Fig. 1A), etiolated *xpf-3* was not hypersensitive to HU treatment alone and showed the same dose-response as wt (Fig. 2A). HU applied to *xpf-3* after UV-B irradiation had a greater effect on the inhibition of hypocotyl elongation, compared to Col-0 wt (Fig. 3A, open symbols). However, the overall pattern was maintained in both Col-0 wt and *xpf-3*, in that HU applied after the two lowest UV-B irradiations induced a similar level of hypocotyl growth inhibition, but there was increased growth inhibition when HU was applied after 10^4 μmol m^{-2} UV-B. The only difference was that *xpf-3* showed an inhibition of hypocotyl elongation after irradiation with 10^3 μmol m^{-2} UV-B only (without subsequent HU treatment) and wt did

not (Fig. 3A, closed symbols). Therefore, the effects of UV-B and HU appear to be additive, acting independently.

Nucleotide excision repair is not required for UV-B -specific gene expression of chalcone synthase

Because *xpf-3* is hypersensitive to UV-B in terms of hypocotyl growth inhibition, and that this sensitivity may be due to an accumulation of unrepaired DNA damage, it is possible that other UV-B -specific responses, such as the expression of *CHS,* are also affected. Using monochromatic UV-B at 290 nm, *CHS* expression was measured in *xpf-3*. In contrast to the hypersensitive response in terms of hypocotyl growth, *CHS* expression in *xpf-3* was not different compared to wt (Fig. 3B). In both Col-0 and *xpf-3*, there was little *CHS* expression in the dark and after 10^2 μmol m^{-2} UV-B. A moderate increase in expression occurred after 10^3 μmol m^{-2} UV-B and about a 2-fold increase in expression after 10^4 μmol m^{-2} UV-B. Again, *xpf-3* began to show hypocotyl elongation inhibition after 10^3 μmol m^{-2} and was strongly inhibited after 10^4 μmol m^{-2} UV-B (Fig. 3A), but *CHS* expression in *xpf-3* remained similar to that in wt.

It may be that 10^4 μmol m^{-2} UV-B irradiation is causing non-specific or general stress responses that include the induction of *CHS* expression (Dixon and Paiva, 1995). If that is the case, then *CHS* expression would likely be higher in *xpf-3* compared to wt; instead, expression in *xpf-3* was not different from wt. Furthermore, adding 1 mM HU only to etiolated seedlings did not affect *CHS* expression in either Col-0 wt or *xpf-3* (Fig. 3B, bottom panel "dark"). Finally, *CHS* expression after UV-B irradiation with subsequent HU treatment was similar in wt and *xpf-3* as was expression after UV-B alone (Fig. 3B).

UV-B hypocotyl growth inhibition is distinct from *UVR8*

The *UVR8* gene encodes a UV-B photoreceptor (Rizzini *et al.,* 2011) responsible for many plant responses to UV-B. However, when etiolated *uvr8-2* mutants were irradiated with UV-B, their hypocotyl growth response was not different from wt (Gardner *et al.,* 2009). Hypocotyl inhibition in response to UV-B in *uvr8-6*, a null mutant

(Favory *et al.,* 2009), was also similar to wt after irradiation with both broadband and monochromatic UV-B (Fig. 4A). Mutants of *COP1* and *HY5* also showed UV-B hypocotyl growth inhibition that was similar to wt (Supplementary Fig. S4). *uvr8* mutants lack *CHS* induction (Kliebenstein *et al.,* 2002; Brown and Jenkins, 2008; Favory *et al.,* 2009), and when etiolated *uvr8-6* mutants were irradiated with UV-B, *CHS* expression was not induced until the fluence reached 10^4 μmol m^{-2} where expression was only about half that of wt (Fig. 4C). Therefore, while inhibition of hypocotyl elongation in response to UV-B does not require *UVR8* in etiolated seedlings, the induction of *CHS* does. In contrast, *UDPgtfp*, a UV-B -specific gene induced independently of *UVR8* (Brown and Jenkins, 2008), was still induced by UV-B in *uvr8* mutants (Fig. 4C).

When HU was applied to *uvr8-6* either alone or after UV-B irradiation, hypocotyl growth inhibition was not different compared to wt (Figs. 2A & 4B) indicating that the cell-cycle response (see below) does not require UVR8. *CHS* expression was not further induced after HU treatment at the lower UV-B fluences; however, it did have a stronger induction than after 10^4 μmol m^{-2} UV-B irradiation alone, although still lower than wt (Fig. 4C). The *UVR8* independent gene, *UDPgtfp*, was also not affected by HU treatment in *uvr8-6* (Fig. 4C). Similar results were seen with *uvr8-2* (Supplementary Fig. S5).

UDPgtfp expression was also measured in *xpf-3* after UV-B irradiation alone and with subsequent HU treatment. After UV-B irradiation only, *UDPgtfp* expression was strongly induced to a similar degree after 10^4 μmol m^{-2} UV-B in both wt and *xpf-3* (Fig. 5A). However, expression was slightly higher in *xpf-3* after irradiation with the lower UV-B fluences, and in the dark (no light treatment). When HU was applied, *UDPgtfp* expression in *xpf-3* was at least 2-fold higher compared to wt in the dark and at the lowest UV-B fluences tested, but expression was similar after 10^4 μmol m^{-2} UV-B (Fig. 5B).

UV-B hypocotyl growth inhibition is caused by cell-cycle arrest

Wt Col-0 seedlings containing a *CYCB1;1-GUS* construct (Colón-Carmona *et al.,* 1999) were irradiated with broadband UV-B, returned to darkness, then harvested 2-48 h after irradiation. *CYCB1;1* is a G2/M -specific gene that is strongly up-regulated in response to DNA damage from ionizing radiation (Culligan *et al.,* 2006). GUS staining

was most prominent at the meristems but also extended into the hypocotyl and cotyledons (Fig. 6A). There was less CYCB1;1-GUS accumulation in dark-grown seedlings overall. Generally, CYCB1;1-GUS accumulation increased over time, peaking around 24 h after UV-B irradiation, and this high level of accumulation persisted until at least 48 h after irradiation. Interestingly, 48 h post-irradiation, staining could be seen along the root and most of the hypocotyl (data not shown).

There was a corresponding induction of *CYCB1;1* expression in Col-0 after 10^4 μmol m^{-2} -UV-B irradiation alone (-HU), but not after the lower UV-B treatments or in the dark (Fig. 6B). This parallels hypocotyl growth inhibition, which was observed after 10^4 μmol m^{-2} but not after 10^2 or 10^3 μmol m^{-2} -UV-B in Col-0 (Fig. 2B). Both *uvr8-6* and *xpf-3* had higher expression of *CYCB1;1* than wt after UV-B irradiation alone at each fluence (Fig. 6B). Expression of *CYCB1;1* was highest in *xpf-3*, which parallels its hypocotyl growth response to UV-B (Fig. 3A). However, the higher expression in *uvr8-6* than wt after each UV-B irradiation (Fig. 6B, -HU) contrasts its hypocotyl response after UV-B irradiation (Fig. 4 A&B). *CYCB1;1* expression was not induced by HU treatment alone in either Col-0, *uvr8-6*, or *xpf-3* (Fig. 6B, +HU). The expression remained similar among all three genotypes when HU was applied after UV-B irradiation, except after 10^4 μmol m^{-2} -UV-B, where *xpf-3* showed the highest expression of *CYCB1;1* (Fig. 6B, +HU).

Discussion

UV-B inhibition of hypocotyl growth is a consequence of cell-cycle arrest initiated by photodimer formation.

Plants have specific photomorphogenic responses to UV-B (Jordan, 2002), including hypocotyl growth inhibition (Kim *et al.,* 1998; Shinkle *et al.,* 2004), changes in gene expression (Ulm *et al*., 2004; Brown *et al*., 2005), and cotyledon expansion (Kim *et al.,* 1998) among others (Gerhardt *et al*., 2005; Barnes *et al.,* 2005; Ulm, 2006). UVR8 is required, along with the transcription factor HY5, for UV-B-specific induction of *CHS* (Ulm *et al.,* 2004; Brown *et al.,* 2005; Brown and Jenkins, 2008). CHS catalyzes the biosynthesis of flavonoids, which is an important element of UV-B light tolerance in

plants (Favory *et al.,* 2009; Gruber *et al.,* 2010). DNA directly absorbs UV-B light, forming photodimers between adjacent bases, and plants have specific DNA repair pathways such as nucleotide excision repair (NER) that remove this type of damage. Responses to DNA damage caused by UV-B light are often not considered photomorphogenic, but rather non-specific, stress-like responses that are also induced by other stimuli (Boccalandro *et al.,* 2001; Brosché and Strid, 2003). However, the formation of photodimers is specific to UV-B light. Here, evidence is provided that the inhibition of hypocotyl growth in response to UV-B irradiation in etiolated *Arabidopsis* is a consequence of cell-cycle arrest that is initiated by photodimer formation.

The inhibition of hypocotyl elongation is a classic photomorphogenic response, and our results with the *xpf-3* mutant (Fig. 1 A&C) indicate that DNA damage, specifically the accumulation of unrepaired photodimers (Fig. 1B), influences this response after UV-B irradiation. The hypersensitivity of *xpf-3* to UV-B irradiation may not be surprising; however, these seedlings are completely viable and can be transferred to white light and to soil, then grown to seed despite the severe inhibition of growth (Fig. S2; Gardner *et al.,* 2009). In etiolated wt Arabidopsis, with functional XPF, there may still be some DNA damage, but the plant is able to maintain cellular processes without growth consequences. At higher UV-B fluences, $\geq$ 30, 000 μmol m^{-2}, DNA damage likely accumulates in wt to a level where seedlings are unable to sustain timely DNA repair, and the hypocotyl growth response approaches that of the NER mutants (Fig. 1A). Therefore, *xpf-3* seedlings may sustain an increased accumulation of photodimers after UV-B irradiation, due to their inability to repair DNA damage, but are in a state of arrested growth until the excess damage is repaired.

XPF is a 5'-endonuclease that mainly functions in NER in plants, but it can also function in mitotic recombination and repair of double strand breaks (Bardwell *et al.,* 1994; Gallego *et al.,* 2000). In addition, it likely has some role in the DNA damage signaling network regulated by the protein kinases ATAXIA-TELANGIECTASIA MUTATED (ATM) and ATM AND RAD3-RELATED (ATR) that recognize double strand breaks and replication blocks, respectively (Garcia *et al.,* 2003; Culligan *et al.,* 2004). Downstream transduction from both ATM and ATR occurs through

SUPPRESSOR OF GAMMA 1 (SOG1), a transcription factor responsible for the expression of several genes induced after γ-irradiation (Yoshiyama *et al.,* 2009). The delayed growth and inhibited transcriptional response to gamma irradiation in *xpf* mutants is reversed in the absence of SOG1 (Pruess and Britt, 2003).

A distinct signaling mechanism for gamma radiation in plants is unlikely due to the almost non-existent levels of gamma radiation experienced on earth. Thus, it seems logical that this signaling pathway would function to maintain genome integrity primarily in response to UV-B irradiation. SOG1 does appear to function in responses to UV-B induced DNA damage since the *sog1-1* mutation reversed the UV-B hypersensitive phenotype of *xpf* (Fig. 1C). This reversal indicates a loss of signal transduction through SOG1 that is either initiated directly from UV-B -specific photodimers or from stalled replication or transcription sites due to photodimer accumulation, a typical result of UV-B light absorption by DNA (Culligan *et al*., 2004; Curtis and Hays, 2007), rather than double-strand breaks. This possible UV-B signaling through SOG1 appears to be independent of ATM and ATR (Supplementary Fig. S3).

Cell-cycle arrest is the ultimate consequence of signaling through SOG1, and it may be responsible for inhibiting the growth of etiolated seedlings after UV-B irradiation. In wt *Arabidopsis* containing a *CYCB1;1-GUS* reporter construct, expression was low in dark-grown seedlings and much higher after UV-B irradiation (Fig. 6A). The accumulation of CYCB1;1-GUS that was sustained until about 48 h after UV-B irradiation is consistent with the time course of hypocotyl elongation inhibition reported by Gardner *et al.* (2009), who showed that hypocotyl growth was inhibited within 6 h after UV irradiation and lasted until 3-4 d later.

The alteration of cell-cycle progression is a known consequence of UV-B light irradiation. Root growth in *atr* mutants is severely inhibited after treatment with replication blocking agents, including UV-B light, due to a loss in regulation of a G2-phase cell-cycle checkpoint (Culligan *et al.,* 2004). *Arabidopsis* mutants more tolerant to UV-B underwent extra rounds of endoreduplication in hypocotyl cells (Hase *et al*., 2006) and were later shown to lack an inhibitor of a complex that promotes cell division (Heyman *et al.,* 2011). Both cell division and elongation contribute to overall growth

(Inzé and De Veylder, 2006). In hypocotyls, the bulk of growth is due to cell elongation with cells that undergo multiple rounds of endoreduplication in light as well as dark (Gendreau *et al*., 1997). A cell-cycle block, especially one that inhibits DNA replication like UV-B light or HU, could conceivably affect elongation and division. Endoreduplication may, in part, be a trigger for cell expansion and elongation (Melaragno *et al*., 1993). Therefore, if endoreduplication is inhibited, elongation may be as well. Cell division is required to initially supply the elongating cells (Gendreau *et al*., 1997), and a disruption in DNA replication could also inhibit this, contributing to an overall inhibition of growth in the hypocotyl after UV-B irradiation.

The photoreactivation experiment shown in Fig. 1D provides further evidence that the inhibition of hypocotyl growth in etiolated seedlings is a consequence of photodimer formation. Based on the report of Hada *et al.* (2000) that the action spectrum of higher plant CPD photolyases has maximum effectiveness at 400 nm, we treated seedlings with 400 nm blue light either during or immediately following the UV-B treatment. While x*pf-3* showed hypersensitivity to UV-B alone, as expected, blue light reversed the mutant phenotype. This suggests that photoreactivation rapidly repairs the photodimers that cannot be repaired by NER in *xpf-3*, and additional inhibition of elongation does not occur.

Inhibition of hypocotyl growth by UV-B is distinct from that caused by HU

To further indicate that a cell-cycle block can result in a similar growth phenotype as UV-B, hydroxyurea (HU) was used to simulate the effects of UV-B irradiation on hypocotyl growth inhibition. HU inhibits DNA replication and induces a G1 cell-cycle block (Planchais *et al*., 2000), and etiolated seedlings treated with HU showed an inhibition of hypocotyl elongation in a dose-dependent manner (Fig. 2A). Although hypocotyl growth was inhibited in etiolated *Arabidopsis* seedlings by both UV-B and HU, their effects appear to be independent. *xpf-3* showed hypersensitivity to UV-B (Fig. 1), but had the same response to HU as wt (Fig. 2A) further suggesting that photodimers may ultimately be responsible. The independent effects of UV-B light and HU on hypocotyl growth inhibition are also clear in that UV-B results in the accumulation of

CYCB1;1, while HU treatment in the dark does not (Fig. 6B). This emphasizes that there may be multiple mechanisms by which hypocotyl growth can be inhibited, since *CYCB1;1* is required at the G2/M transition and HU blocks the cell cycle at the G1/S transition.

UV-B -specific expression of *CHS* was unaffected by both UV-B and HU in wt and *xpf-3* (Fig. 3B). Since the *xpf-3* mutant and wt both have intact UVR8, UV-B -specific *CHS* expression would not be expected to be different from wt unless photodimer formation had some effect on *CHS* expression. This also showed that the UV-B irradiation and HU treatment themselves did not simply induce a general stress response in *xpf-3* that resulted in increased *CHS* expression (Dixon and Paiva, 1995).

Inhibition of hypocotyl growth of etiolated seedlings by UV-B is largely independent of *UVR8*

The UV-B -specific hypocotyl growth inhibition in etiolated seedlings is not merely a phytotoxic effect from DNA damage but a photomorphogenic response that occurs largely independently of the *UVR8* photoreceptor (Fig. 4A). There has been at least one report of two distinct UV-B photomorphogenic pathways, where DNA was implicated as the chromophore in one of them (Shinkle *et al.,* 2004). UV-B induced signaling pathways that are independent of UVR8 have also been reported (Brown and Jenkins, 2008; Wargent *et al*., 2009; González Besteiro *et al.,* 2011) and further indicate that other UV-B perception mechanisms are present in plants. Brown and Jenkins (2008) described a high-fluence rate response that likely overlaps with oxidative stress or wound signaling pathways that induced gene expression specifically in response to UV-B irradiation, but did not require *UVR8*. UVR8 was shown to be necessary for normal leaf development and expansion in response to UV-B irradiation through regulation of endoreduplication and stomatal differentiation, but reduced cell divisions in the leaf epidermis were not dependent on UVR8 (Wargent *et al*., 2009). The mitogen-activated protein kinase (MAPK) stress-response cascade that functions in reactive oxygen species (ROS) signaling (Mittler *et al.,* 2011) also functions in UV-B responses (Holley *et al.,* 2003; Kalbina and Strid, 2006). Specifically, the MAP kinase phosphatase 1 (MKP1)

pathway is activated after UV-B irradiation but independently of *UVR8* (González Besteiro *et al.,* 2011). Oxidative stress can be an accompanying problem when irradiating green, photosynthetic tissue with UV-B light due to a disruption of electron transport through photosystem II (Jansen *et al*., 1996; Vass *et al*., 1996). General stress pathways are activated by this disruption and may explain the activation of MAPK signaling processes after UV-B irradiation that function in response to ROS production (González Besteiro *et al.,* 2011).

UDPgtfp was one of the *UVR8* independent, UV-B -specific genes previously reported (Brown and Jenkins, 2008). This particular UDP-glucosyltransferase is rapidly induced by H_2O_2 and glycosylates the auxin indole-3-butyric acid (IBA) to regulate growth and physiological responses to biotic and abiotic stress (Tognetti *et al.,* 2010). Our results confirmed its UV-B -specific induction independent of *UVR8* (Fig. 4C). The interplay of ROS formation and signaling with UV-B responses was not directly tested here. However, because etiolated tissue was used in these experiments, ROS formation, at least resulting from disrupted photosynthesis, should be minimal. The higher expression of *UDPgtfp* in the *xpf-3* mutant (Fig. 5) may reveal a novel function of this gene in the DNA damage response from blocked replication that leads to cell-cycle arrest, although expression due to ROS formation and signaling cannot be ruled out.

Inhibition of hypocotyl elongation by UV-B via cell-cycle arrest is a property of etiolated seedlings

We report that *uvr8* shows inhibition of hypocotyl growth by UV-B that is similar to wt (Figs. 4, S1, S5), which is an apparent contradiction to previously documented *uvr8* phenotypes. It is important to distinguish that our growth conditions of complete darkness with pulses of UV-B light are quite different from other studies that showed *uvr8* mutants grown under continuous white light conditions, either with or without supplementary UV-B light, lacked the UV-B-induced hypocotyl growth inhibition of wt (Favory *et al*., 2009). Also, overexpression of UVR8 resulted in hyper-induction of *CHS* along with increased hypocotyl growth inhibition to UV-B light (Favory *et al*., 2009), where here the

hypersensitive UV-B hypocotyl growth observed in *xpf-3* was not accompanied by enhanced *CHS* induction (Fig. 3).

As noted in our initial studies (Gardner *et al.*, 2009), we chose to use completely etiolated plants to reduce the possibility of detecting events that are induced by other, non-UV-related, photoreceptors and to eliminate complicating factors that might be associated with de-etiolation, such as chlorophyll and other screening pigment production or the synthesis of the photosynthetic apparatus. Therefore, it is difficult to directly compare our fluence-response sensitivity with that reported by others. For example, Favory *et al.* (2009) measured growth inhibition in light-grown plants after 4 d of continuous UV-B treatment at 1.5 μmol m^{-2} s^{-1} and corresponds to a total fluence of about 5 x 10^5 μmol m^{-2}. They also reported experiments with 1 h or 6 h of UV-B at 1.5 μmol m^{-2} s^{-1}, resulting in 5.4 x 10^3 and 3.24 x 10^4 μmol m^{-2} total UV-B. This is on the same order of the experiments reported here at 10^4 μmol m^{-2}, which were given over 16 min for the broad band source or 52 min at 290 nm with the monochromator.

Our results are also different from the original isolation of *uvr8* that reported it more sensitive to UV-B irradiation than wt (Kliebenstein *et al*., 2002). *uvr8* sensitivity is more pronounced in plants that have had an "acclimation" period to low levels of UV-B supplied with continuous white light (González Besteiro *et al.,* 2011) and is consistent with the lack of *CHS* expression in *uvr8* mutants (Kliebenstein *et al.,* 2002; Brown and Jenkins, 2008; Favory *et al.,* 2009). Therefore, a sensitive phenotype in light-grown *uvr8* plants may be a result of damage from a lack of flavonoids to screen the UV-B. Our measurements in etiolated seedlings are taken before an effect from the induction of flavonoid biosynthesis can be observed (Supplementary Fig. S1). *CHS* expression in the etiolated *uvr8* mutants (Fig. 4C & Fig. S5), however, is consistent with previous reports, regardless of growth conditions (Kliebenstein *et al*., 2002; Brown and Jenkins, 2008; Favory *et al*., 2009).

Another possible explanation for the UV-B inhibition seen in wt but not in *uvr8* by others (Favory *et al.,* 2009) may be due to the increase in flavonoid synthesis induced by UV-B. It has long been known that flavonoids can inhibit auxin transport (Stenlid, 1976; Jacobs and Rubery, 1988; Gardner and Sanborn, 1989), and this inhibition of auxin

transport could result in inhibition of hypocotyl elongation in the wt. In *uvr8*, flavonoid accumulation would not occur in response to UV-B, and auxin transport and growth would not be inhibited. A similar explanation may apply to the slight hyposensitivity that we sometimes observe in *uvr8* at low fluences of UV-B (Fig. 4A). At 10^3 μmol m^{-2} UV-B there is only slight inhibition of growth; whereas, the same fluence caused a substantial increase in *CHS* expression in the wt (Fig. 4C). Perhaps the CHS-derived flavonoids in the wt cause a slight inhibition of growth at very low fluences. These would be absent in *uvr8*. Testing this hypothesis on the relative contribution of flavonoids and auxin transport is beyond the scope of the present study but will be the subject of future investigation.

In conclusion, the results presented here show that there is an underlying pathway specific to plant responses to UV-B, distinct from signal transduction through *UVR8,* that influences early *Arabidopsis* seedling growth shortly after germination. This pathway appears to originate from UV-B -induced DNA photodimers and results in photomorphogenic responses such as hypocotyl growth inhibition through a disruption in the cell cycle.

Figures

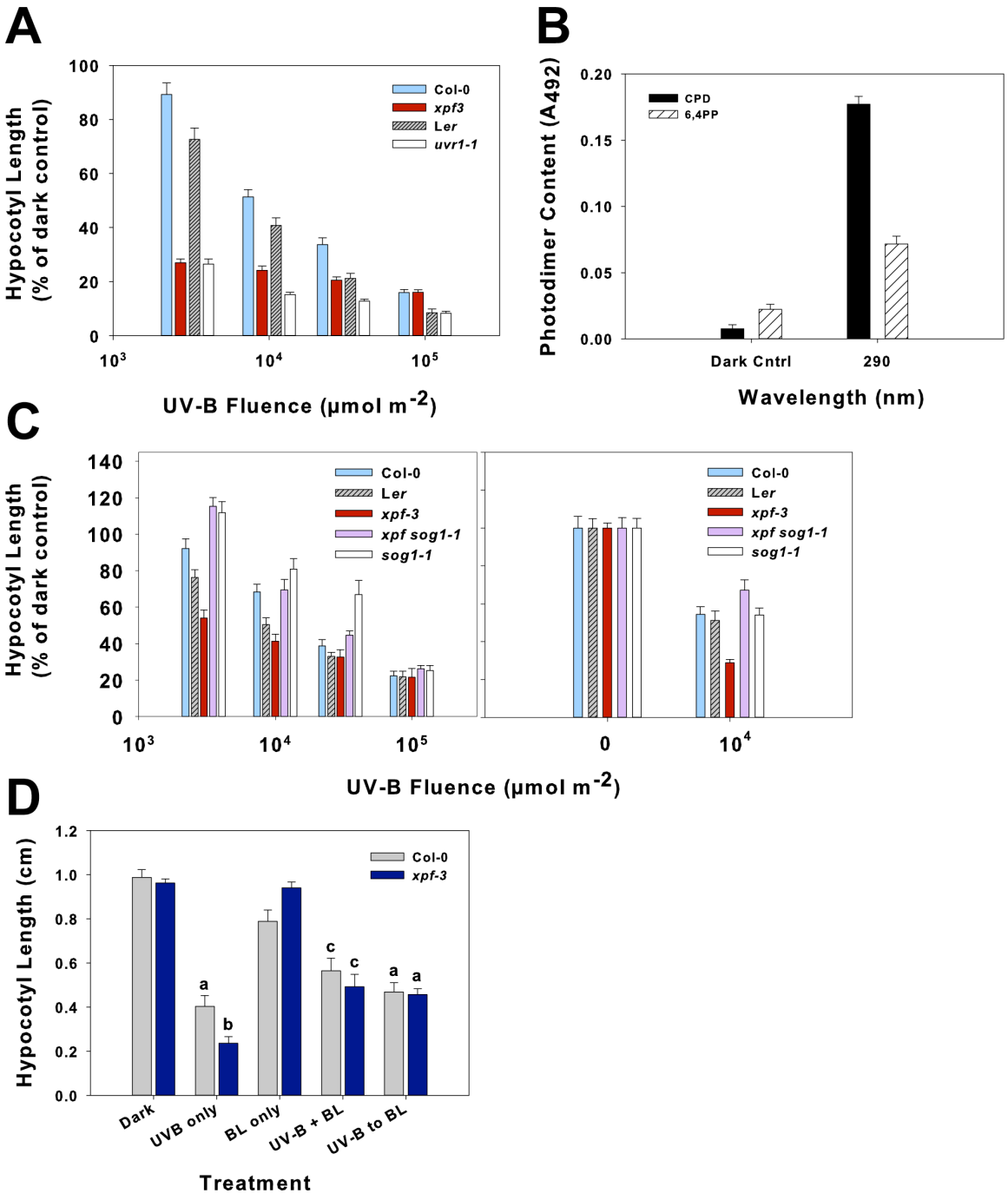
A
Hypocotyl Length (% of dark control)
Col-0
xpf3
Ler
uvr1-1
UV-B Fluence (µmol m-2)
B
Photodimer Content (A492)
CPD
6,4PP
Dark Cntrl
290
Wavelength (nm)
C
Hypocotyl Length (% of dark control)
Col-0
Ler
xpf-3
xpf sog1-1
sog1-1
UV-B Fluence (µmol m-2)
D
Hypocotyl Length (cm)
Col-0
xpf-3
Dark
UVB only
BL only
UV-B + BL
UV-B to BL
Treatment

Figure 1. Hypocotyl growth responses to UV-B and blue light and photodimer accumulation in etiolated *Arabidopsis* seedlings. Fluence response for inhibition of hypocotyl growth by UV light in *Arabidopsis* mutants deficient in DNA repair or DNA damage signaling and photodimer content in wild type Col-0 after UV-B irradiation. **A)** Fluence response curves for nucleotide excision repair (NER) mutants, *xpf-3* (Col-0) and *uvr1-1* (L*er*). Two-day-old etiolated seedlings were irradiated with the total fluence indicated and returned to the dark for two additional days. Data are expressed as percent of the unirradiated dark control of the same genotype (± S.E.). **B)** CPD and 6,4PP content in etiolated Col-0 irradiated with 10^4 μmol m^{-2} monochromatic UV-B at 290 nm. Content is expressed as mean absorbance at 492 nm ± S.E. (n=6). **C)** Fluence response curves for *xpf-3*, *xpf sog1-1* (Col-0/L*er*) and *sog1-1* (Col-0) irradiated with either broadband (left graph) or narrowband (right graph) UV-B. Treatments and measurements were as described in **A)**. **D)** Photoreactivation of UV-B -induced hypocotyl growth inhibition in Col-0 and *xpf-3* seedlings. Two-day-old etiolated seedlings were irradiated either with UV-B at 290 nm, blue light at 400 nm (BL), UV-B at 290 nm and BL at 400 nm concurrently (UV-B + BL) or UV-B followed by BL irradiation (UV-B, BL), returned to darkness and photographed two days later. Total UV-B fluence was 10^4 μmol m^{-2}, and total BL treatment fluence was ~8,000 μmol m^{-2} over the same duration as the UV-B irradiation (approximately 52 min). Means are displayed ± S.E. and letters indicate significance ($p<0.05$) based on a Student's t-test comparing Col-0 wt and *xpf-3* and treatments.

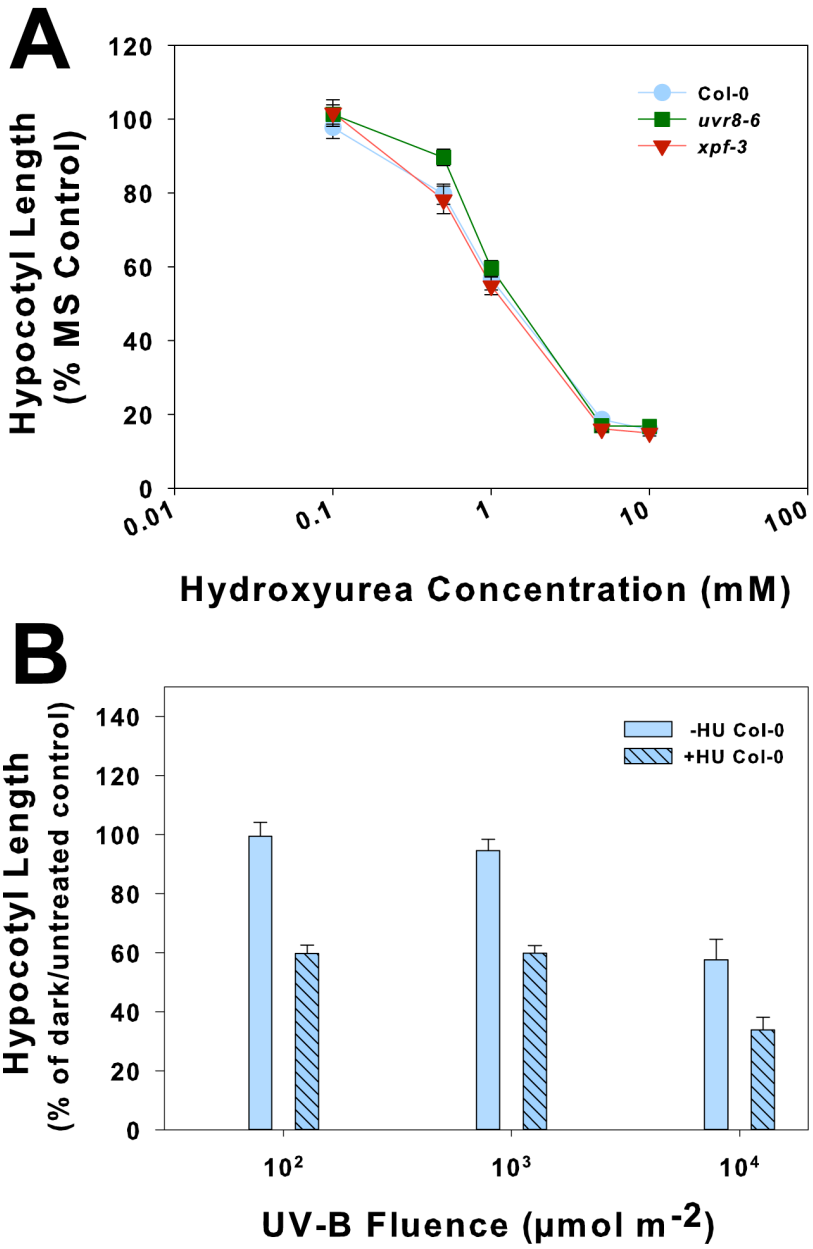

Figure 2. Hypocotyl growth inhibition after hydroxyurea (HU) treatment in *Arabidopsis* seedlings. Two- to three-day-old etiolated seedlings were either **A)** treated with HU over a range of concentrations (Col-0 wt, *uvr8-6* and *xpf-3*) or **B)** irradiated with narrow band UV-B (290 nm) with (+HU) or without (-HU) the addition of 1 mM HU after irradiation (Col-0 wt only). Seedlings were returned to the dark after treatments for two additional days. Data are expressed as percent of a 0.5X MS-treated only or untreated dark control (± S.E.).

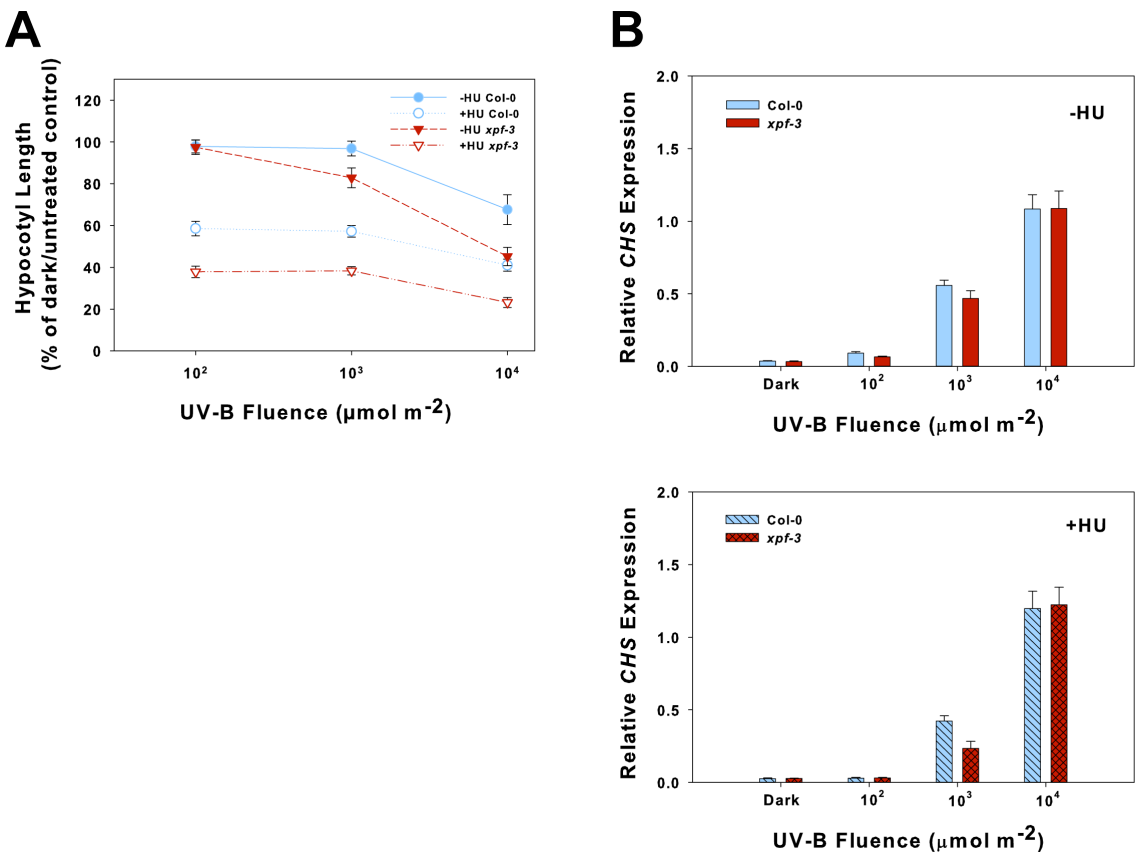

Figure 3. Effect of hydroxyurea (HU) after UV-B irradiation in the nucleotide excision repair (NER) mutant *xpf-3*. A) Hypocotyl growth inhibition in two- to three-day-old etiolated seedlings irradiated with UV-B and subsequently treated with 1 mM HU. Circles represent Col-0 wt and triangles represent *xpf-3*. Filled symbols indicate response after UV-B irradiation only (-HU); open symbols indicate response after UV-B irradiation with 1 mM HU treatment (+HU). Data are expressed as percent of the untreated dark control of the same genotype (± S.E.). **B)** UV-B -specific chalcone synthase (*CHS)* expression in two- to three-day-old etiolated seedlings irradiated with UV-B at 290 nm. Seedlings were placed back in the dark and harvested 2 h later. Expression (± SE; n= 3) was determined by quantitative real-time PCR using the Livak $2^{-\Delta\Delta CT}$ method with *ACTIN2* as the reference gene. Top panel shows expression after UV-B irradiation only (-HU). Bottom panel shows expression after UV-B irradiation with 1 mM HU treatment (+HU).

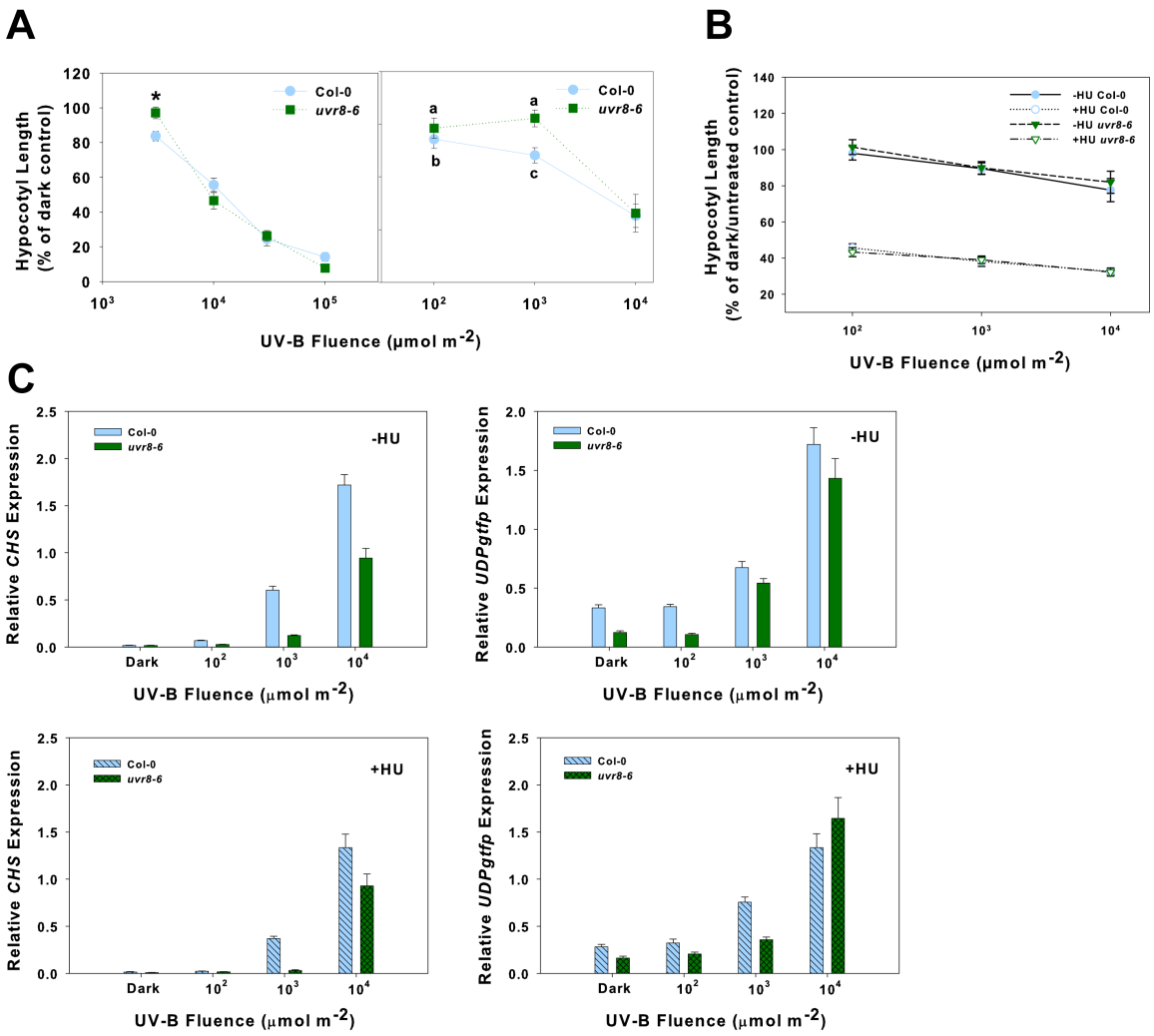
A
B
C
Hypocotyl Length (% of dark control)
Col-0
uvr8-6
UV-B Fluence (µmol m-2)
Hypocotyl Length (% of dark/untreated control)
-HU Col-0
+HU Col-0
-HU uvr8-6
+HU uvr8-6
Relative CHS Expression
Relative UDPgtfp Expression
-HU
+HU
Dark

Figure 4. Effect of UV-B irradiation and hydroxyurea (HU) on hypocotyl growth and gene expression in *uvr8-6*. A) Fluence response curve for inhibition of hypocotyl growth with either broad-band UV-B (left panel) or narrow band UV-B at 290 nm (right panel). **B)** Hypocotyl growth inhibition in etiolated seedlings irradiated with UV-B and subsequently treated with 1 mM HU. Circles represent Col-0 wt and triangles represent *uvr8-6.* Filled symbols indicate response after UV-B irradiation only (-HU); open symbols indicate response after UV-B irradiation with 1 mM HU treatment (+HU) and lines for Col-0 and *uvr8-6* are superimposable for this response. Growth experiments in **A)** and **B)** used two- to three-day old etiolated seedlings at time of treatment. Data are expressed as percent of the untreated dark control of the same genotype (± S.E.); asterisk (*) indicates significance ($p<0.05$) based on a Student's t-test comparing Col-0 wt and *uvr8-6* at $3x10^3$ μmol m^{-2} UV-B; letters indicate significance ($p<0.05$) based on a Student's t-test between all pair-wise comparisons of Col-0 wt and *uvr8-6* at 10^2 and 10^3 μmol m^{-2} UV-B treatments. **C)** UV-B specific gene expression in two- to three-day-old etiolated seedlings irradiated with UV-B at 290 nm. Seedlings were placed back in the dark and harvested 2 h later. Expression (± SE; n= 3) was determined by quantitative real-time PCR using the Livak $2^{-\Delta\Delta CT}$ method with *ACTIN2* as the reference gene. Top panels show expression after UV-B irradiation only (-HU). Bottom panels show expression after UV-B irradiation with 1 mM HU treatment (+HU). Left panels: *CHS*; right panels: *UDPgtfp*.

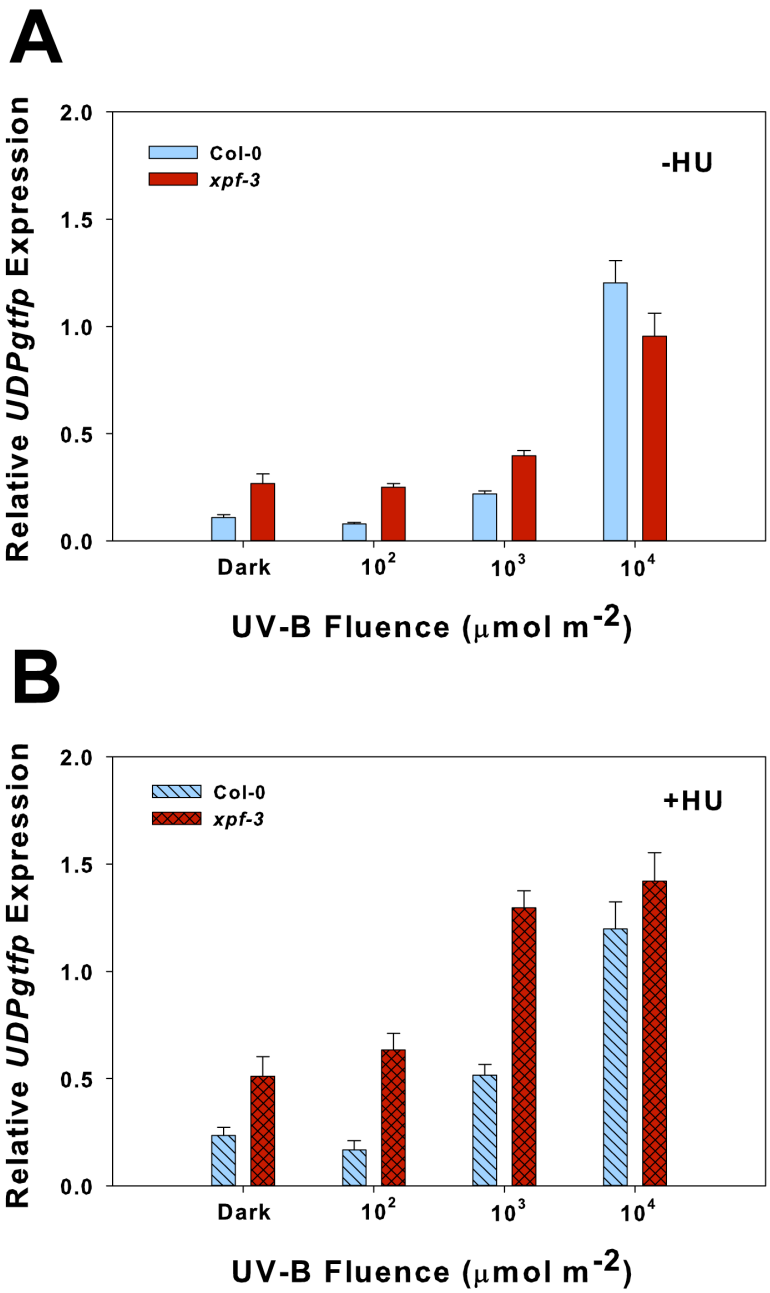

Figure 5. Expression of the UVR8-independent gene *UDPgtfp* in the nucleotide excision repair mutant *xpf-3* and wt after HU treatment. Two- to three-day-old etiolated seedlings were irradiated with UV-B at 290 nm with 1 mM HU added immediately after irradiation. Seedlings were placed back in the dark and harvested 2 h later. Expression (± SE; n= 3) was determined by quantitative real-time PCR using the Livak $2^{-\Delta\Delta CT}$ method with *Actin2* as the reference gene. **A)** UV-B irradiation only (-HU); **B)** UV-B irradiation with 1 mM HU treatment (+HU).

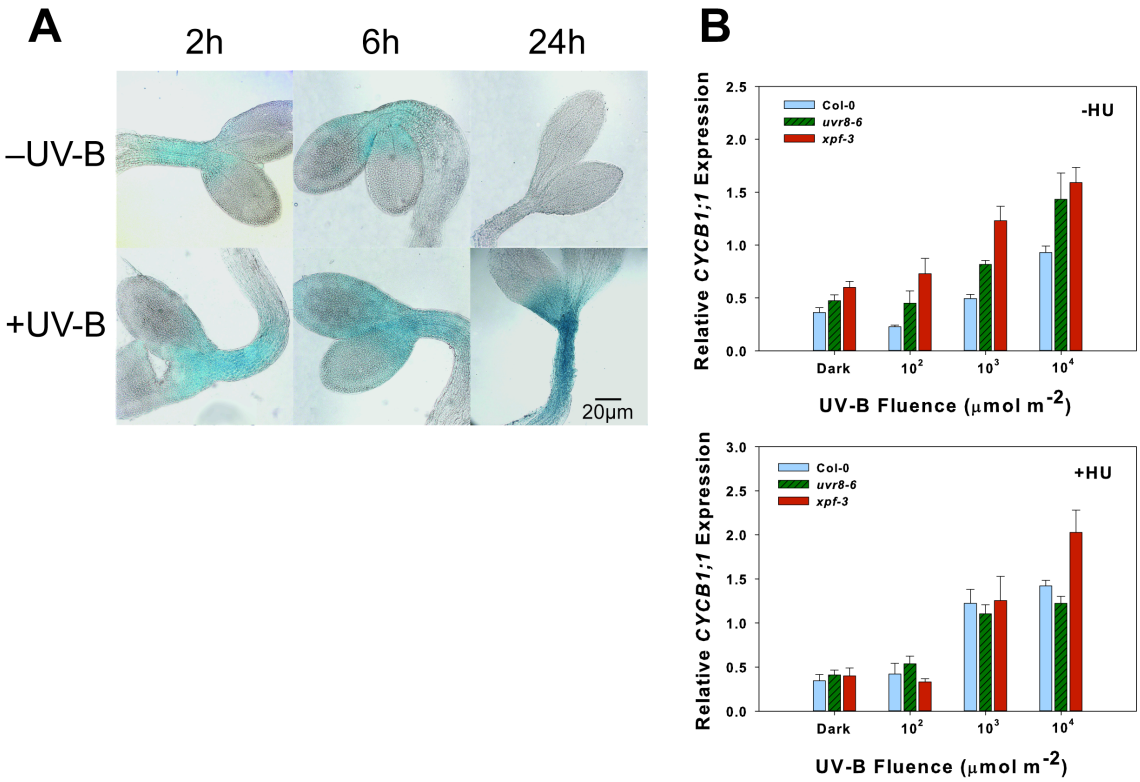

Figure 6. Expression of *CYCB1;1* in etiolated seedlings irradiated with UV-B. A) CYCB1;1-GUS accumulation in two- to three-day-old etiolated Col-0 seedlings irradiated with 10^4 μmol m^{-2} broadband UV-B. Approximately ten seedlings were observed from each time point. Photographs show representative samples. **B)** *CYCB1;1* expression in Col-0*, uvr8-6,* and *xpf-3* 24h after 10^2, 10^3, or 10^4 μmol m^{-2} UV-B light irradiation at 290 nm. "Dark" samples indicate dark/unirradiated control (-HU) and 1mM HU treated/unirradiated control (+HU).

Supplementary Figures

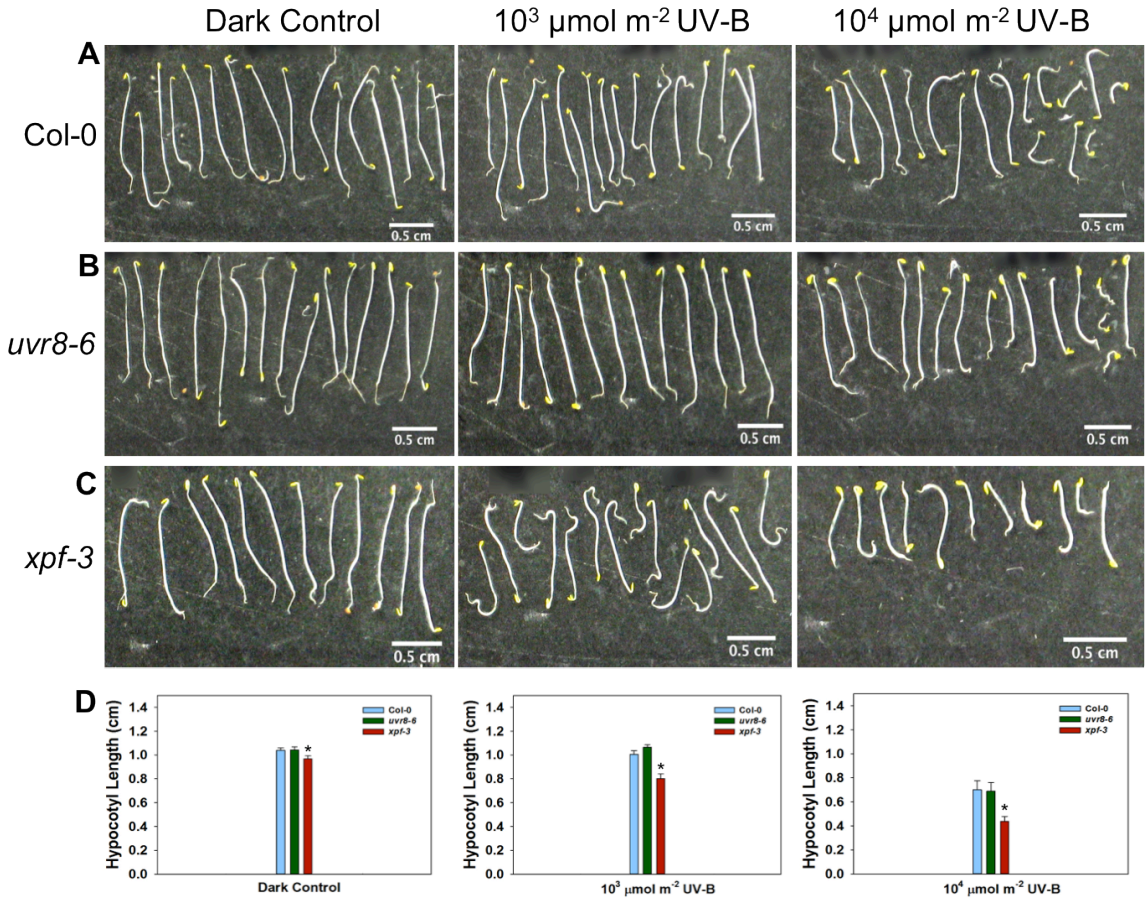

Figure S1. Response of etiolated *Arabidopsis* seedlings to monochromatic UV-B irradiation. A-C) Hypocotyl growth inhibition in two- to three-day-old etiolated seedlings irradiated with narrow band UV-B at 290 nm. Seedlings were returned to darkness after UV-B irradiation and photographed two days later. **D)** Mean hypocotyl lengths (cm) of the seedlings shown for each treatment (± S.E.). Asterisks (*) denote significance ($p<0.05$) based on a Student's t-test comparing Col-0 wt and each mutant.

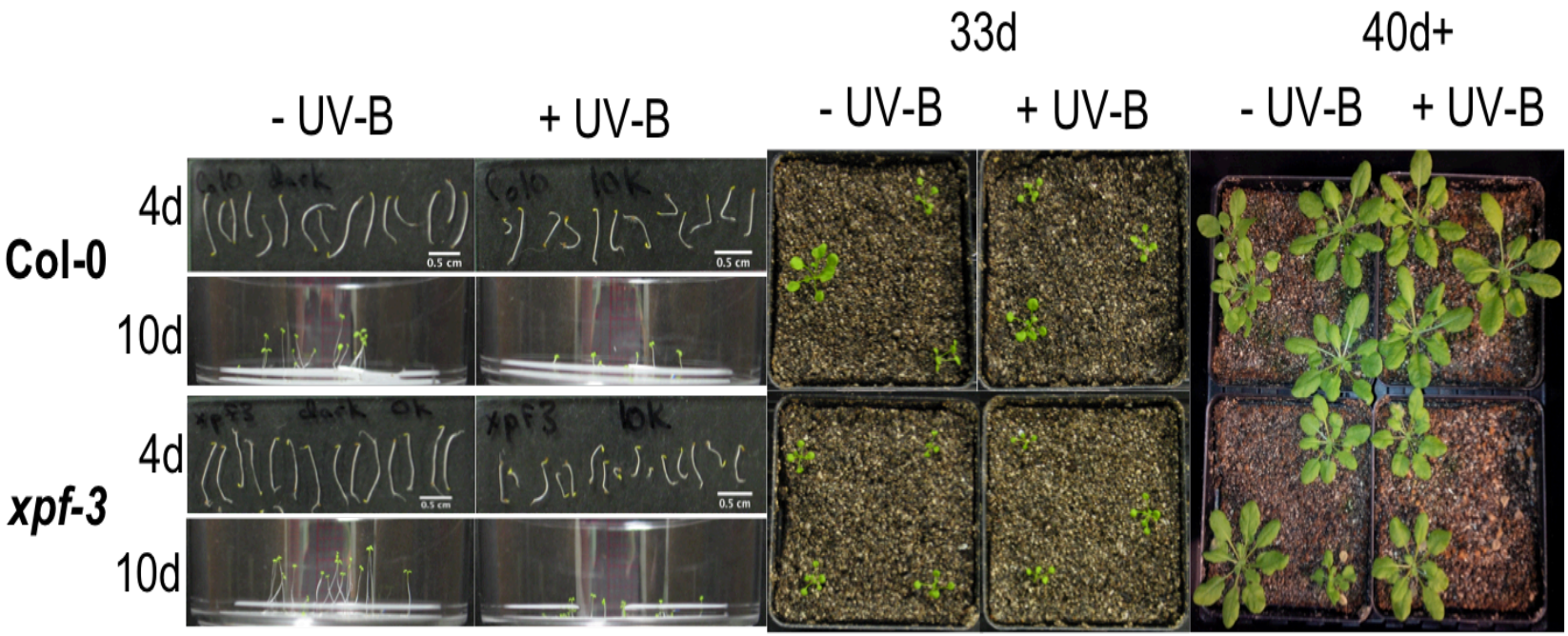
33d
40d+
- UV-B
+ UV-B
- UV-B
+ UV-B
- UV-B
+ UV-B
Col-0
4d
10d
xpf-3
4d
10d
0.5 cm
0.5 cm
0.5 cm
0.5 cm

Figure S2. Growth of Col-0 and *xpf-3* after UV-B irradiation. Two- to three-day-old etiolated seedlings were irradiated with 10^4 μmol m^{-2} broadband UV-B light. Seedlings were returned to darkness after UV-B irradiation. One set was photographed two days later (4d) and another set of seedlings, with or without initial UV-B irradiation, were grown in Petri dishes under 80 μmol m^{-2} s^{-1} cool-white fluorescent lamps with a 8:16 light:dark cycle for ~10d. At that point, plants were transferred to soil and placed back in the same light conditions under Mylar due to potential UV sensitivity of *xpf-3*. Approximately 5 weeks after transfer to soil, plants were switched to flowering conditions (16:8 light:dark cycle) and grown to seed.

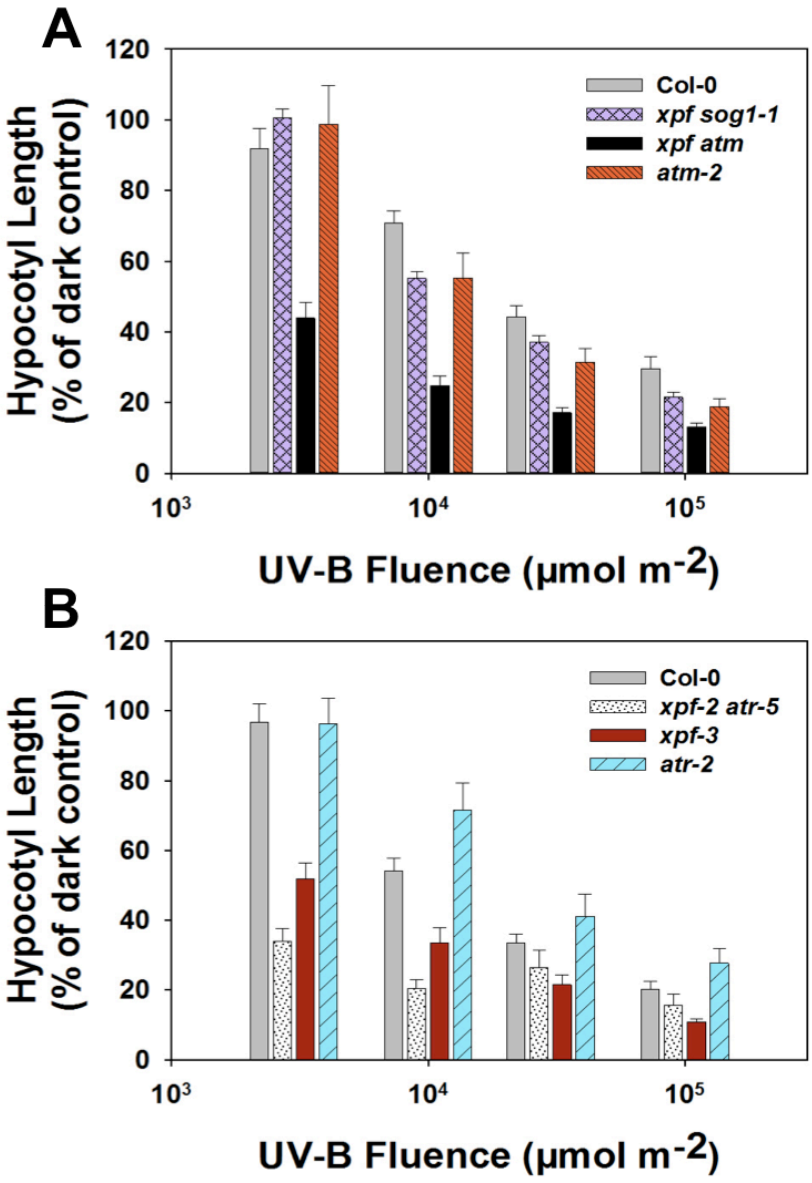

Figure S3. UV-B fluence response of hypocotyl growth inhibition in DNA damage response mutants. Two- to three-day-old etiolated seedlings were irradiated with broad-band UV-B. Seedlings were returned to darkness after UV-B irradiation and photographed two days later. Data are expressed as percent of the untreated dark control of the same genotype (± S.E.). **A)** *xpf sog1-1*, *xpf atm,* and *atm* (SALK_040423C) *Arabidopsis* mutants. *xpf atm* displays a *xpf-3* hypersensitive UV-B phenotype regarding hypocotyl growth inhibition and is unlike *xpf sog1-1* and the single *atm* mutant that are similar to wt. **B)** *xpf-2 atr-5*, *xpf-3*, and *atr-2* (SALK_032841C) *Arabidopsis* mutants. *xpf-2 atr-5* mutant also has a *xpf-3* hypersensitive UV-B phenotype regarding hypocotyl growth inhibition, where *atr-5* is similar to wt. Double mutants *xpf atm* and *xpf-2 atr-5* were provided courtesy of Dr. Anne Britt (UC-Davis, CA, USA).

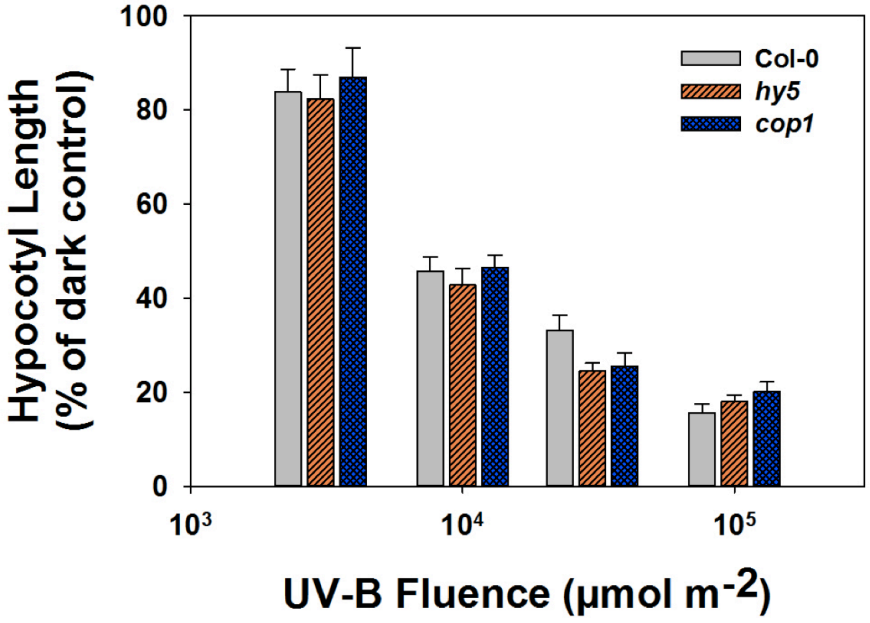

Figure S4. UV-B fluence response of hypocotyl growth inhibition in *hy5* and *cop1*. Two- to three-day-old etiolated seedlings were irradiated with broadband UV-B. Seedlings were returned to darkness after UV-B irradiation and photographed two days later. Data are expressed as percent of the untreated dark control of the same genotype (± S.E.). HY5 and COP1 are components in the UVR8 photoreceptor signaling pathway in response to UV-B. Mutants of *hy5* and *cop1* have similar hypocotyl growth inhibition as wt after UV-B irradiation.

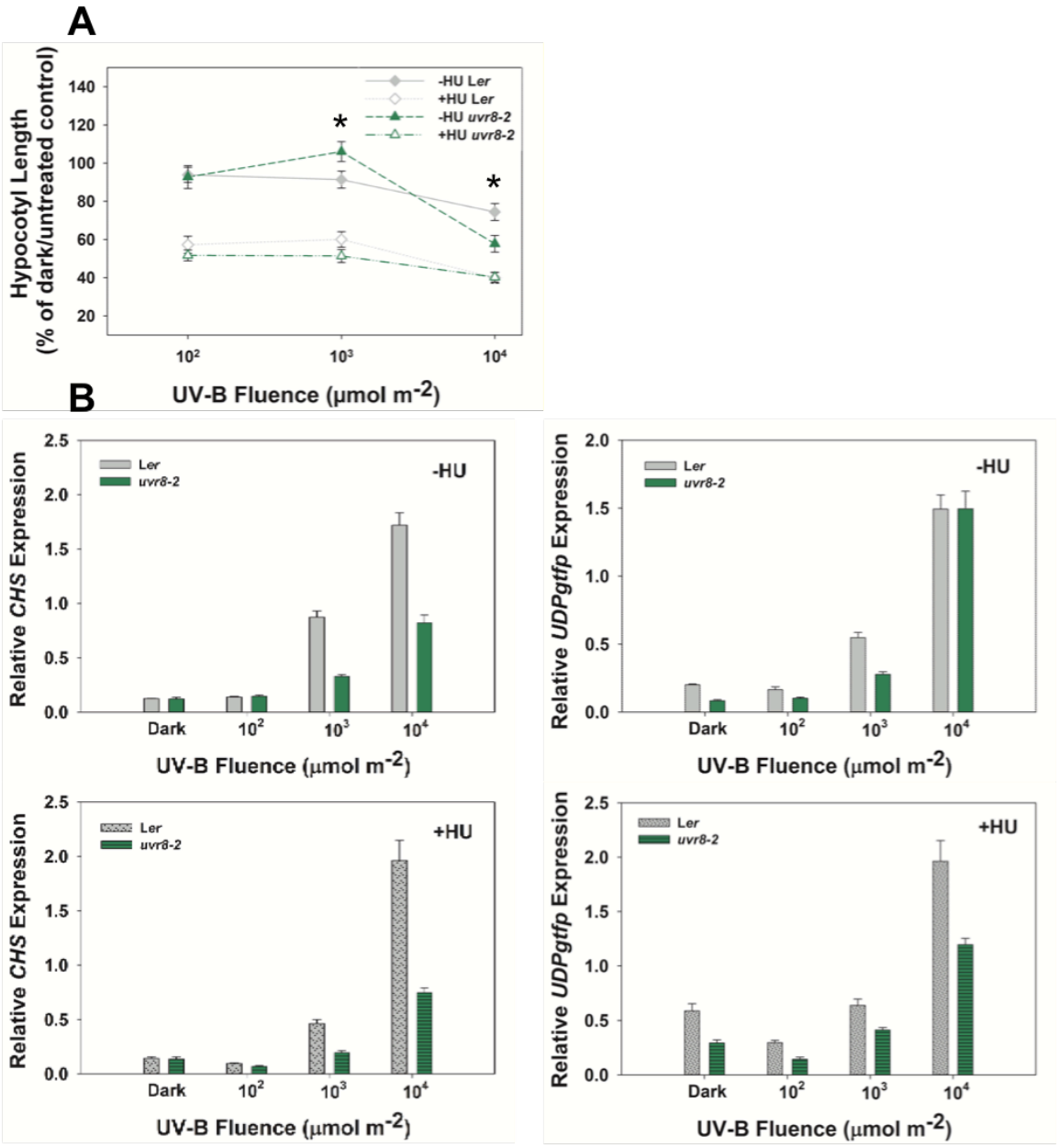
A
Hypocotyl Length
(% of dark/untreated control)
-HU Ler
+HU Ler
-HU uvr8-2
+HU uvr8-2
UV-B Fluence (µmol m-2)
B
Relative CHS Expression
Relative UDPgtfp Expression
Ler
uvr8-2
-HU
+HU
Dark
UV-B Fluence (µmol m-2)

Figure S5. Effect of UV-B irradiation and hydroxyurea (HU) on hypocotyl growth and gene expression in *uvr8-2*. A) Hypocotyl growth inhibition in two- to three-day-old etiolated seedlings irradiated with narrow band UV-B at 290 nm and subsequently treated with 1 mM HU. Circles represent L*er* wt and triangles represent *uvr8-2*. Filled symbols indicate response after UV-B irradiation only (-HU); open symbols indicate response after UV-B irradiation with 1 mM HU treatment (+HU). Data are expressed as percent of the untreated dark control of the same genotype (± S.E.), asterisks (*) indicate significance ($p<0.05$) based on a Student's t-test between L*er* wt and *uvr8-2* at each fluence (in the absence of HU). **B)** UV-B -specific gene expression in two- to three-day-old etiolated seedlings irradiated with UV-B at 290 nm. Seedlings were placed back in the dark and harvested 2 h later. Expression (± SE; n= 3) was determined by quantitative real-time PCR using the Livak $2^{-\Delta\Delta CT}$ method with *ACTIN2* as the reference gene. Top panels show expression after UV-B irradiation only (-HU). Bottom panels show expression after UV-B irradiation with 1 mM HU treatment (+HU). Left panels: *CHS*; right panels: *UDPgtfp*.

Chapter 3

Conclusion

Research leading to knowledge regarding how plants perceive and respond to UV-B light has made substantial progress in the last few years, especially with the characterization of UVR8 as a UV-B photoreceptor and further definition of its signaling pathway. While UVR8 no doubt plays a major role in UV-B photoperception (Christie *et al*., 2012), it cannot explain or account for all UV-B responses observed in plants (Gardner *et al*., 2009; Wargent *et al*., 2009; González Besteiro *et al.,* 2011; Biever *et al*., 2013). The research findings presented in this dissertation adds to information regarding UV-B photobiology in plants by documenting an alternative pathway of UV-B perception and signaling in plants that is independent of UVR8. This work is unique in that it provides evidence that UV-B photomorphogenesis, specifically hypocotyl growth inhibition, can be influenced by the direct absorption of UV-B light by DNA.

In Chapter 2, it was shown that hypocotyl growth inhibition in etiolated *Arabidopsis* seedlings appears to, ultimately, be the consequence of UV-B-induced photodimer formation. The NER mutants of *Arabidopsis*, *uvr1-1* and *xpf-3*, were hypersensitive in terms of hypocotyl growth inhibition to UV-B, and photodimers accumulated in Col-0 wt *Arabidopsis* after UV-B irradiation. In addition, the hypocotyl growth inhibition response in *xpf-3* was photoreactivatable, meaning that blue light treatment or activation of the photolyases decreased the hypocotyl growth sensitivity. The accumulation of *CYCB1;1* after UV-B irradiation was an indication that cell-cycle arrest occurred, which was likely initiated by photodimer formation. Therefore, cell-cycle arrest provided the basis for hypocotyl growth inhibition. These results are important because they provide evidence that a photomorphogenic response, the inhibition of hypocotyl growth in etiolated *Arabidopsis* seedlings, is influenced by UV-B-specific DNA damage and does not require UVR8. In addition, they reinforce the idea that multiple UV-B perception mechanisms exist in plants and may be more analogous to UV-B perception in human cells. The parallels to UV-B perception in humans were how initial UV-B perception hypotheses were formed for plants (Caldwell, 1971). These results also

provide evidence that a UV-B perception pathway initiated by UV-B-specific DNA damage can influence photomorphogenic growth in plants, rather than being a general response that is not necessarily specific to UV-B or part of UV-B-specific signaling.

The distinction between the response measured in the research presented here and that induced by UVR8 is further demonstrated in the data presented in the Appendices. In Appendix A, *uvr8* mutants grown under continuous light conditions either with or without supplementary UV-B for 5 days lacked UV-B-induced hypocotyl growth inhibition, which is consistent with what would be expected in a photoreceptor mutant. The fact that there is a difference in *uvr8* UV-B hypocotyl growth inhibition between light-grown and etiolated seedlings could be described by the UVR8-dependent synthesis of flavonoids. As suggested at the end of Chapter 2, flavonoids can inhibit auxin transport and may be responsible for hypocotyl growth inhibition in plants exposed to any type of light. Therefore, wild type *Arabidopsis* under continuous white light (WL) + UV-B would have increased synthesis of flavonoids induced by UV-B light, but *uvr8* mutants would not. The increased amount of flavonoids in wild type would then cause hypocotyl growth inhibition, while this inhibition would not be observed in *uvr8*. Without continuous light treatment or light exposure in etiolated seedlings as described in this work, hypocotyl growth was variable in the different wild types and *uvr8* mutants, but overall growth and response to UV-B was similar. Since our light treatments are given as brief pulses and in etiolated seedlings, the hypocotyl growth measurements are taken before the synthesis and accumulation of flavonoids would have an affect on UV screening. Furthermore, data presented in Appendix B shows that etiolated *rup* mutants, negative regulators of the UVR8 perception pathway, are also not affected in hypocotyl growth inhibition. And finally, UV-B-induced hypocotyl growth inhibition is also independent of the PhyA photoreceptor, as was also shown previously (Gardner *et al*, 2009).

Apart from expanding knowledge about how plants perceive UV-B light, specifically, this work also has implications toward the understanding of initial development of germinating seedlings and how light signals may ultimately affect plant growth. Initially, a germinating seedling extending out of the soil will have minimal

synthesis of flavonoids due to the lack of prior light exposure. It will have little protection from the first sunlight exposure, and therefore, it will be more vulnerable to UV-B light. UV-B light, present in solar radiation, is more readily absorbed by DNA at this stage and would lead to photodimer formation. The recognition of photodimers occurs by DNA repair enzymes involved in either NER or photoreactivation and ultimately initiates downstream processes that require the transcription factor SOG1, which eventually leads to growth inhibition through cell-cycle arrest. UVR8 is required for UV-B-dependent production of flavonoids and, as the plant continues to grow, is important for protection from UV-B light. However, the UV-B perception pathway initiated by the direct absorption via DNA is still relevant as some UV-B light passes through the leaf and reaches the inner cellular components. These two pathways are distinct UV-B perception mechanisms, operating in tandem, to influence plant growth.

XPF is an enzyme that is involved in NER, but it functions in other DNA repair processes as well. Mutants in several other components involved inof NER and the photolyase enzymes themselves do not show the same level of UV-B hypersensitivity seen in *xpf-3* and *uvr1-1*, indicating that there is something unique about the endonucleases. It could be that these enzymes are important for recognition and downstream DNA damage signaling processes. Even though these enzymes may function in more general growth responses, they have specific responses to UV-B light, which further reinforces the idea that UV-B is important to overall growth processes. The ultimate regulation or influence on the cell cycle is a particularly interesting aspect of this research. Exploration into how more precisely UV-B or other environmental stimuli controls it and the other components involved is an area that I would like to pursue in the future.

Future Directions

To fully characterize the pathway proposed from research presented in this dissertation, several approaches could be applied. One would be to document the changes from seed to seedling on a molecular level in hypocotyls using a combination of transcriptomic, proteomic, and metabolomic approaches first in wild type *Arabidopsis* and then with comparison to *xpf-3* mutants. These data could be supplemented with

observed cellular changes in wild type vs. *xpf-3* hypocotyls using powerful techniques, such as confocal microscopy. After initial development, the transition from dark to light-grown seedlings could also be documented in a similar manner, with emphasis on UV-B light-induced changes in wild type vs. *xpf-3*. In order to know better whether UV-B induced hypocotyl growth inhibition in etiolated *Arabidopsis* is quantitatively related to photodimer formation, a more sophisticated technique for detecting and quantifying photodimers is needed, such as LC-MS methods (Douki *et al*. 2000). CYCB1;1 is only one particular cyclin of many in addition to dozens of other proteins that are involved in regulating the cell cycle. Other components could be monitored to substantiate cell-cycle arrest, such as cyclin dependent kinases (CDKs) or WEE1, through protein levels or gene expression. The core proteins that are required for initial recognition of DNA damage based on studies in yeast and humans are XPC, Rad23B, XPA, RPA, TFIIH, and CENTRIN2, and they would also have to be analyzed to provide a comprehensive view of the exact steps from direct photodimer detection to cell-cycle arrest or other downstream effects. Plants contain genetic homologs of all of the listed proteins except XPA. There has been limited research on their biochemical functions in plants to determine whether they play a similar role to what has been defined in other systems. However, the specific link between DNA damage recognition and ultimate downstream consequences remains to be discovered. As mentioned, inhibited auxin transport could be a contributing factor to hypocotyl growth inhibition after UV-B irradiation. Auxin also influences the cell cycle, so measuring auxin transport would provide more insight into the regulation of the UV-B-induced hypocotyl growth response through possible interference from flavonoids (Brown *et al*., 2001) or direct effects on the cell cycle.

The affects of light on plant growth and development are incredibly complex. Characterizing plant perception of UV-B and subsequent responses is an important part in understanding how plants respond to their light environment, in general. The understanding gained from this work may help researchers better predict how changes in the light environment, such as potential increased fluxes of UV-B, will affect plant growth to better determine how plants will respond overall and adapt to a changing environment.

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Appendix A

A. Additional data on *uvr8* - Visit to the laboratory of Dr. Roman Ulm at the Université de Genève

Previous results showing that the *uvr8-2* mutant responded like wt after UV-B irradiation with regard to hypocotyl growth inhibition were unexpected (Gardner *et al*., 2009; Chapter 2, Fig. S5). The opportunity arose to visit Dr. Roman Ulm's lab to conduct experiments with several *uvr8* mutant lines and assess their hypocotyl growth response to UV-B irradiation. One major distinction between our results and the Ulm lab's results is that they use continuous light treatments and we use brief pulses of light in etiolated seedlings. While I was in Geneva, I measured hypocotyl growth inhibition in three *uvr8* mutants that were exposed to continuous white light (WL) either with or without continuous UV-B light. I also conducted fluence response experiments in etiolated seedlings to measure hypocotyl growth inhibition after WL or UV-B irradiation. The WL was delivered with Osram (Lumilux) L18W/380 warm white fluorescent lamps with a fluence-rate of 3.5 μmol m^{-2} s^{-1}. The UV-B light was given via Philips TL20W/01-RS lamps with a fluence of 0.05-0.06 mW m^{-2}, which corresponds to a fluence-rate around 1.5 μmol m^{-2} s^{-1} calculated for 312 nm. These conditions are similar to those described in Favory *et al*. (2009). Lower UV-B wavelengths were excluded with 304 nm cut-off filters for all UV-B treatments.

Hypocotyl growth inhibition of *uvr8* mutants was determined using the standard conditions that the Ulm lab uses to distinguish UV-B sensitivity in *uvr8* and wt (Ulm *et al*., 2004; Oravecz *et al*., 2006; Favory *et al.*, 2009). Seeds were sown on filter paper treated with ½ MS and GA, in this case it was GA_3, and were put directly under WL or WL + UV-B as described above. Hypocotyls were measured after 5 days under each light treatment. Hypocotyl lengths are presented as a percentage of those grown under WL only (Fig. A-1). All wild type accessions, L*er*, Col, and Ws, had ~ 40-50% inhibition with UV-B treatment where the *uvr8* mutants had only ~20-30% inhibition with UV-B treatment. This is consistent with previous results reporting that mutants are less sensitive to UV-B with regard to hypocotyl growth inhibition (Favory *et al*., 2009). This is also the

type of response one would expect for a mutant of a photoreceptor. However, to my knowledge this insensitivity to UV-B has only been shown under these particular continuous light conditions and highlights the differences we see in *uvr8* mutants under our conditions (Chapter 2: Figs. 4&S5), which is likely a different response than what is shown here.

Fluence response curves were also conducted in etiolated *uvr8* mutants treated with either WL or UV-B, but with lower fluences than what we typically use. This was based on Dr. Roman Ulm's suggestion that even our lowest fluence $3x10^3$ μmol m^{-2} may be too much for etiolated seedlings. The fluences here ranged from 10^2 μmol m^{-2} – $3x10^3$ μmol m^{-2} for both WL and UV-B. Overall, the hypocotyl growth inhibition under WL and UV-B varied considerably, and there was no distinct response dependent on fluence (Fig. A-2). There was considerable growth promotion under WL and UV-B in Col and *uvr8-6*, where hypocotyl lengths were 20-60% longer than the dark controls, which may be due to poor germination of the dark controls for these genotypes. There is variation with the middle two UV-B treatments in Col and *uvr8-6,* but the inhibition after the lowest and highest fluences is about the same (Fig. A-2B). Similarly, the $3x10^3$ μmol m^{-2} fluence did not inhibit hypocotyl elongation more than the 10^2 μmol m^{-2} fluence in L*er* and *uvr8-2*; however, the middle two fluences seemed to have more of an inhibitory effect in *uvr8-2* than L*er* (Fig. A-2B). Despite the high variability in these results, this work was the basis for routinely using 10^2-10^4 μmol m^{-2} UV-B irradiations in our subsequent experiments, which can be seen in most of the data presented in Chapter 2. Along with the data in Chapter 2, we concluded that the hypocotyl growth inhibition response observed in etiolated seedlings is different from the hypocotyl growth inhibition response seen here in seedlings grown under continuous light (Fig. A-1). We further concluded that our conditions are not simply inducing a stress response where we are unable to observe "photomorphogenic" differences between *uvr8* and wt.

Figures

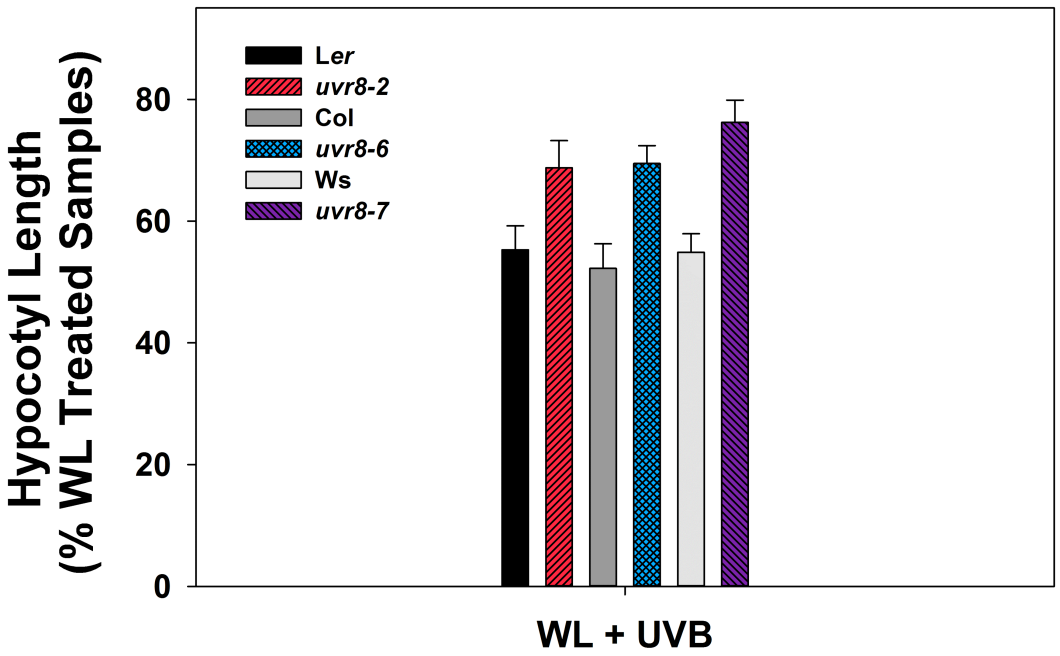

Figure A-1. Hypocotyl growth inhibition in *uvr8* mutants grown under continuous white light with UV-B. Seeds were broadcast on filter paper treated with ½ MS and GA_3 solution and placed in either **A)** continuous white light (WL) with a fluence-rate of 3.5 µmol m^{-2} s^{-1} or **B)** continuous WL with supplementary UV-B (above 304 nm) with a fluence-rate of ~1.5 µmol m^{-2} s^{-1} for 5 days. Ten seedlings from each treatment were transferred to a glass plate and photographed. Hypocotyls were measured using ImageJ and data are expressed as a percent of dark controls (± SE).

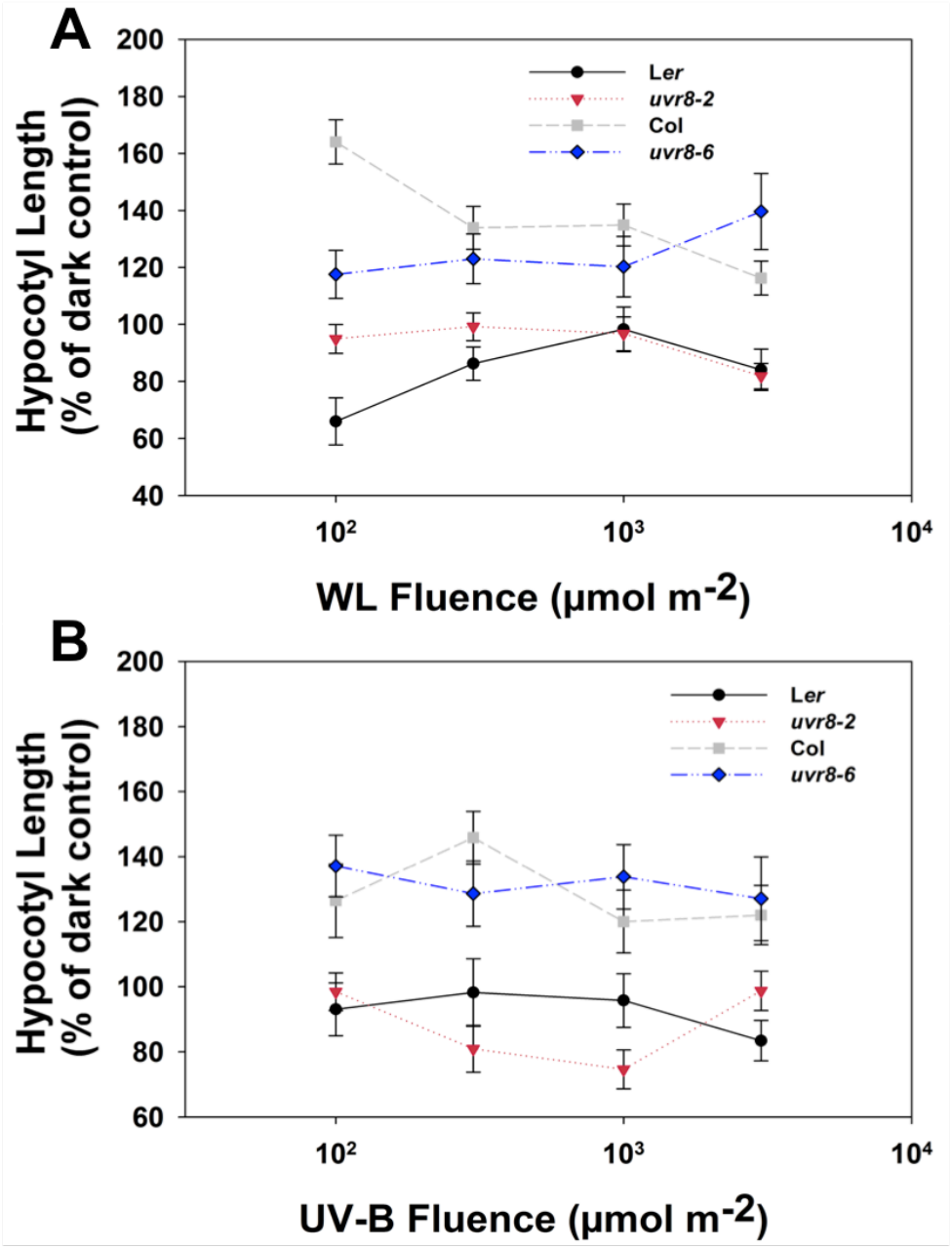
A
Hypocotyl Length
(% of dark control)
200
180
160
140
120
100
80
60
40
Ler
uvr8-2
Col
uvr8-6
10²
10³
10⁴
WL Fluence (μmol m-2)
B
Hypocotyl Length
(% of dark control)
200
180
160
140
120
100
80
60
Ler
uvr8-2
Col
uvr8-6
10²
10³
10⁴
UV-B Fluence (μmol m-2)

Figure A-2. Fluence response curves of *uvr8* mutants to white light (WL) or UV-B irradiation. Seeds were broadcast on filter paper treated with ½ MS and GA_3 solution and placed in darkness until germination. Seedlings were irradiated with either **A)** white light (WL) with a fluence-rate of 3.5 µmol m^{-2} s^{-1} or **B)** UV-B (above 304 nm) with a fluence-rate of ~1.5 µmol m^{-2} s^{-1}. Total fluence was achieved by varying the duration of light treatment from approximately 1-30 min based on the fluence-rate for both WL and UV-B. Seedlings were placed back in darkness after irradiation for two additional days. At that time, seedlings were transferred to a glass plate and photographed. Hypocotyls were measured using ImageJ and data are expressed as a percent of dark controls (± SE).

Appendix B

B. UV-B fluence response curves of other mutants involved in photomorphogenesis

Gardner *et al*. (2009) previously assessed the UV-B fluence response in several photoreceptor mutants of the phytochromes, cryptochromes, and phototropins. They found that the response to UV-B light in etiolated seedlings was not dependent on any of the known photoreceptors. However, we were not sure whether this was true for other responses, such as the induction of chalcone synthase (*CHS*). It was suggested that the *CHS* expression we see after our treatments could be dependent on phytochrome A, since it is known to also absorb in the UV range. Therefore, I measured hypocotyl growth inhibition and *CHS* induction after UV-B irradiation at 290 nm. The inhibition of hypocotyl growth elongation in *phyA* is similar to the Col-0 wt (Fig. B-1A), as previously reported with broadband UV-B (Gardner *et al*., 2009). Expression of *CHS* was similar in *phyA* and wt in samples harvested immediately and 2 h after irradiation (Fig. B-1B). However, there was higher *CHS* expression in *phyA* at all other time points after irradiation (Fig. B-1B). This appears to be a difference in the kinetics of how *CHS* is induced in each genotype, which may or may not be specific to UV-B. Since expression was essentially the same at the peak of expression after irradiation (2 h), we concluded that *CHS* expression was not dependent on PhyA in our system.

Recently, two homologous proteins were identified in *Arabidopsis* that negatively regulate the UVR8 signaling pathway (Gruber *et al*., 2010) and were called REPRESSOR OF UV-B PHOTOMORPHOGENESIS 1 and 2 (RUP1 and RUP2). The *rup* mutants showed increased expression of *HY5* and *CHS* with a higher accumulation of anthocyanins and increased hypocotyl growth inhibition than wt in response to UV-B irradiation (Gruber *et al*., 2009). We obtained *rup* T-DNA insertion lines available through ABRC to determine their response to UV-B. We found no difference in hypocotyl elongation inhibition between any of the *rup1* and *rup2* T-DNA insertion mutants and wt using our standard fluence response curve conditions (Fig. B-2). This

further demonstrates that the hypocotyl growth response of etiolated seedlings to UV-B light occurs independently of UVR8 and its signaling pathway.

Figures

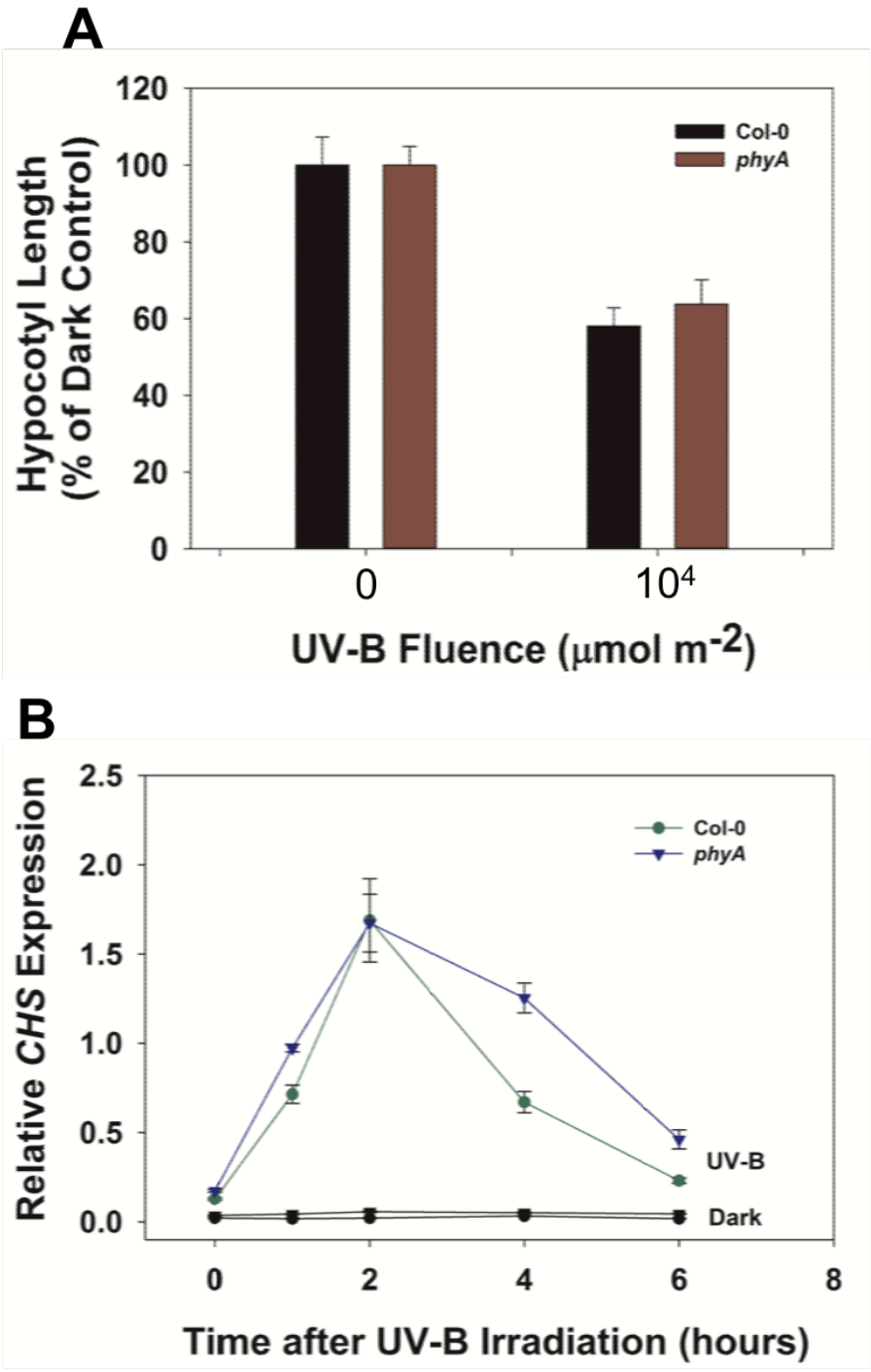
A
Hypocotyl Length
(% of Dark Control)
120
100
80
60
40
20
0
Col-0
phyA
0
10^4
UV-B Fluence (μmol m^-2)
B
Relative CHS Expression
2.5
2.0
1.5
1.0
0.5
0.0
Col-0
phyA
UV-B
Dark
0
2
4
6
8
Time after UV-B Irradiation (hours)

Figure B-1. UV-B fluence response curve and induction of *CHS* in *phyA*. A) Hypocotyl growth inhibition in two- to three-day-old etiolated seedlings irradiated with narrow band UV-B at 290 nm. Data are expressed as percent of the untreated dark control of the same genotype (± S.E.). **B)** UV-B -specific *CHS* expression in two- to three-day-old etiolated seedlings irradiated with UV-B at 290 nm. Seedlings were placed back in the dark and harvested immediately (0 h) to 6 h after irradiation ("UV-B"), each sample had a corresponding dark control ("Dark"). Expression (± SE; n= 3) was determined by quantitative real-time PCR using the Livak $2^{-\Delta\Delta CT}$ method with *ACTIN2* as the reference gene.

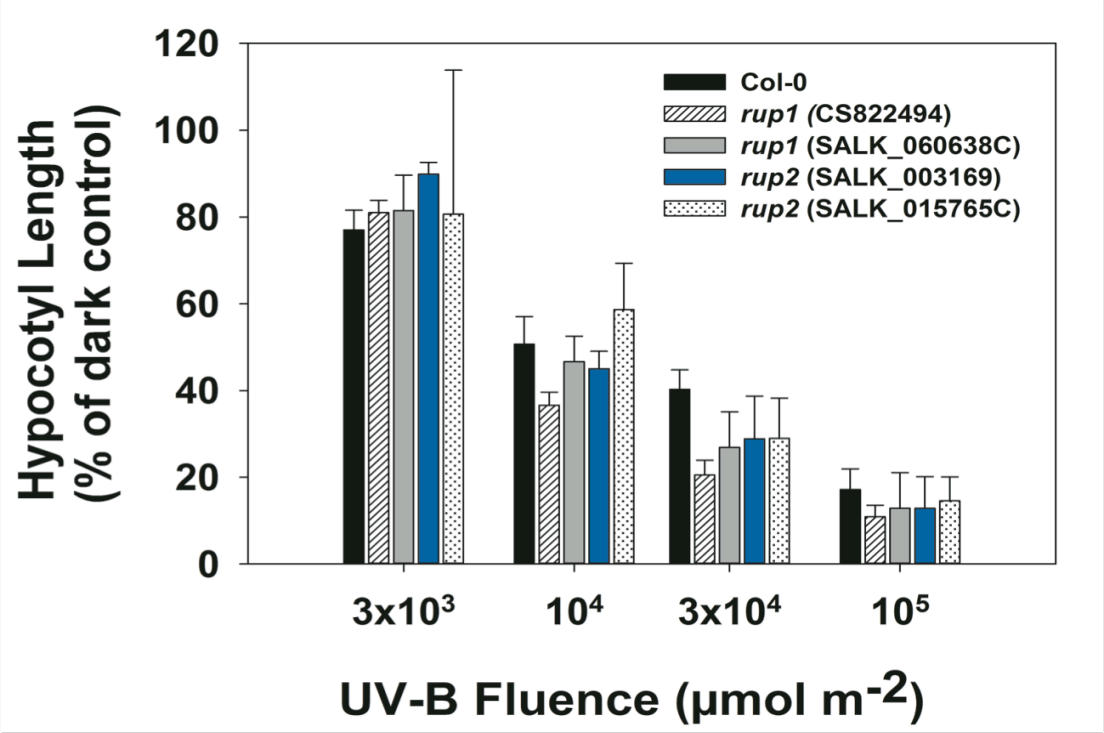

Figure B-2. UV-B fluence response curves of *rup* mutants. Hypocotyl growth inhibition in two- to three-day-old etiolated seedlings irradiated with broadband UV-B. Data are expressed as percent of the untreated dark control of the same genotype (± S.E.).